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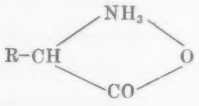
XXVIII. NOTE ON TYROSINE.

By ARTHUR GEAKE and MAXMILIAN NIERENSTEIN.

From the Biochemical Laboratory, Chemical Department, University of Bristol.

(*Received June 9th, 1915.*)

Reference has already been made to a preliminary observation that tyrosine is not methylated by diazomethane in ethereal suspension [Geake and Nierenstein, 1914, 1, p. 292]. The present communication confirms this observation, which is of interest both for the chemistry of methylo-caseinogen [Geake and Nierenstein, 1914, 1], since this substance gives Millon's reaction, and for that of tyrosine. The fact that the carboxyl group of tyrosine is not methylated is quite in accordance with our experiences with other amino acids [Geake and Nierenstein, 1914, 2] which have led us to accept the "betaine" formula [Meyer and Jacobson, 1913]

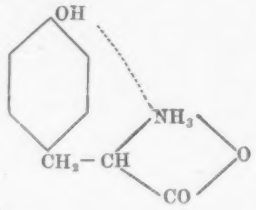


for amino acids. It has further also been found that an hydroxyl-group in para-position to a nitrogen atom is not methylated by diazomethane, as for example in the cases of γ-pyridone, chelidamic and cuminaminic acids [Pechmann, 1895, Meyer, 1906, 1 and 2]. These substances yield unchanged γ-pyridone, the dimethyl ester of chelidamic acid and the methyl ester of methoxy-cuminaminic acid.

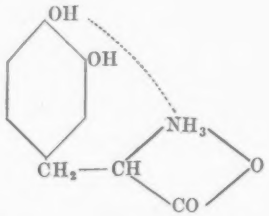
Meyer expresses the influence of the nitrogen atom on the hydroxyl group by dotted lines, but emphasises at the same time as follows: "das Wesen der Erscheinungen wird aber natürlich durch derlei Zeichensprache nicht im mindestens enthüllt und man kann nur den in jüngster Zeit wiederholt

gezogenen Schluss rekapitulieren, dass die derzeit herrschenden Anschauungen über Valenz nicht ausreichen, die Gesamtheit der Beobachtungen zu erklären." In this connection reference ought also to be made to the classical investigations of Kaufmann [1911] on valency with which our observations are in full accord.

The following formula therefore expresses the behaviour of tyrosine towards diazomethane:



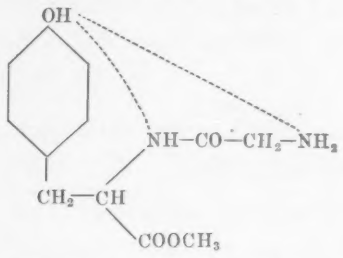
In view of the fact that the hydroxyl group in the meta-position of cuminaminic acid undergoes methylation when treated with diazomethane an experiment was also made with 3:4-dihydroxyphenylalanine [Funk, 1911] to which the formula:



is assigned. The reaction was accompanied by a strong evolution of nitrogen, which indicated that apparently methylation had taken place, especially as nitrogen is not evolved when tyrosine is treated with diazomethane. The material kindly sent by Dr Funk was, however, so small in quantity that we were not able to isolate any definite product. We intend to repeat the experiment on a larger scale later on, since dihydroxyphenylalanine is now more accessible through its isolation from *Vicia faba* by Guggenheim [1913].

Since the carboxyl groups of the amino acids and the peptides become accessible to methylation on acylation, when the "betaine"-ring is opened [Geake and Nierenstein, 1914, 2], the action of diazomethane on glycyl-tyrosine was also investigated especially with the view of studying the behaviour of the tyrosine hydroxyls in caseinogen towards diazomethane. The product thus obtained was the glycyl-tyrosine methyl ester, as we were

able to recover on hydrolysis glycyl-tyrosine and on oxydation *p*-hydroxybenzoic acid. It is apparent that the nitrogen atoms of the glycyl-tyrosine



continue to have an influence on the hydroxyl-group, which leads us, at least for the time-being, to the conclusion *that the tyrosine hydroxyls in methylocaseinogen are not methylated by diazomethane.*

Experimental.

Action of diazomethane on l-*tyrosine.* One g. tyrosine from hair (m.p. 303–306°) was treated with 0·5 g. (2 mol.) diazomethane; no visible reaction took place and 0·85 g. unchanged tyrosine (m.p. 303°) was recovered. In another experiment 0·2 g. tyrosine from caseinogen and 0·1 g. (2 mol.) diazomethane were used and 0·2 g. unchanged tyrosine (m.p. 306°) recovered.

Action of diazomethane on dl-3 : 4-*dihydroxyphenylalanine.* 0·2 g. dihydroxyphenylalanine was treated with 0·25 g. (4 mol.) diazomethane. The substance dissolved with evolution of gas, leaving a residue (0·04 g.) which after recrystallisation from alcohol and water formed small needles which melted at 225°. It is possible that this was unchanged dihydroxyphenylalanine for which the following melting points are given: 238° (Funk) and 280° (Guggenheim). The ethereal solution gave an oil which we have not investigated.

*Action of diazomethane on glycyl-*l-*tyrosine.* One g. glycyl-*l*-tyrosine prepared according to Abderhalden and Oppler [1907] was treated with 1 g. diazomethane; on evaporation of the ether a solid was left, which crystallised from methyl alcohol in small needles and melted at 123–124°. The *glycyl-*l-*tyrosine methyl ester* thus obtained dissolves easily in alcohol, acetone and to some extent also in water. If the ester is melted, cooled and then remelted the melting point rises to about 295–300°, glycyl-*l*-tyrosine anhydride being formed during the melting process as in the case of leucyl-*l*-tyrosine [Fischer and Schrauth, 1907].

Analysis: 0·1488 g.: 13·2 cc. N_2 (moist) 19°, 772 mm.
0·3038 g.: 27·3 cc. N_2 (moist) 16°, 770 mm.
0·1206 g.: 0·2546 g. CO_2, 0·0722 g. H_2O.
0·1222 g.: 0·2558 g. CO_2, 0·0672 g. H_2O.

		Cal. for $C_{12}H_{16}O_4N_2$	
C	.. 57·60, 57·09 %	C	.. 57·14 %
H	.. (8·43), 7·06 %	H	.. 6·35 %
N	.. 10·32, 10·58 %	N	.. 10·94 %

Hydrolysis of glycyl-l-tyrosine methyl ester. One g. of the ester was dissolved in 10 cc. alcohol and warmed on the water bath with 2·5 cc. N/10 alcoholic potash. It was then diluted with 10 cc. water and acidified with 2·5 cc. N/10 hydrochloric acid and the solid filtered off. The product consisted of glycyl-*l*-tyrosine and glycyl-*l*-tyrosine anhydride, which were separated by shaking with dilute hydrochloric acid. The anhydride was crystallised from water and melted at 293–297°. The glycyl-*l*-tyrosine recovered from the hydrochloride crystallised from dilute alcohol in needles, melting at 183°, the melting point not being depressed by glycyl-*l*-tyrosine; mixed melting point found, 182–183°.

Oxidation of glycyl-l-tyrosine methyl ester. Two g. of the ester were dissolved in 20 cc. of 30 per cent. potassium hydroxide and boiled for 3–4 hours with an excess of potassium permanganate. When acidified with hydrochloric acid a small precipitate was obtained which after several recrystallisations from water formed small prismatic needles, melting at 213–215° (m.p. of *p*-hydroxybenzoic acid, 213–215°). A mixed melting with this acid gave 213–215°. For further identification 0·3 g. of the oxidation product was also converted with dimethyl sulphate and alcoholic potash into anisic acid (m.p. found, 186°; m.p. of anisic acid, 184°).

In conclusion we wish to thank Dr Casimir Funk for the *dl*-3 : 4-dihydroxyphenylalanine kindly sent us and also the Bristol University Colston Research Committee for a grant which defrayed the expenses of this investigation.

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XXIX. THE ORIGIN OF THE GLUCOSAMINE OBTAINED IN THE HYDROLYSIS OF *BOLETUS EDULIS*.

By WINIFRED ROSS (*Carnegie Scholar*).

From the Chemical Research Laboratories, United College of St Salvator and St Leonard, University of St Andrews.

(*Received June 22nd, 1915.*)

The existence of chitin in fungus cellulose has long been known, but the question of the origin of the glucosamine obtained in the hydrolysis of dried fungus extract has given rise to two distinct views. The first view is that the glucosamine residue is pre-existent, either as glucoproteins, or as glucosamine glucosides. It has been proved by Müller [1901] that glucoproteins give on hydrolysis a carbohydrate compound identical with that obtained from chitin. The second theory is, that glucosamine is an adventitious product, formed in the process of hydrolysis by the interaction of carbohydrate residues and amino acids.

From the results of numerous experiments, strong evidence in favour of the first theory has been obtained. In particular, reference must be made to the recent isolation of definite glucosamine glucosides by Yotake and Sera [1912]. The material used in their investigations was a species of Lycoperdon, (*L. gemmatum, Batsch*), and, by hydrolysis with concentrated mineral acid, they obtained a compound, termed by them "lycoperdin," which from its reactions proved to be an amino glucoside containing two glucosamine residues. Still more recently, they obtained the same compound from other species of Lycoperdon (*L. bovista*), and also from *Geaster granulosus*.

With the object of isolating similar glucosides, the following research was carried out on *Boletus edulis*. This particular fungus was selected, as it was already known that a large proportion of fungus cellulose existed in the plant, and, therefore, it seemed a probable source of amino glucosides.

Investigations on *Boletus edulis* had previously been carried out by Reuter [1912], who examined various extracts, but more particularly those yielding amino acids. He finally obtained a fraction containing the carbohydrate residues, but did not examine it in detail. It was accordingly decided to examine this carbohydrate fraction for the presence of amino glucosides.

In the extraction of the fungus material, Reuter's experimental method was carefully followed, and thereafter the examination of the dried extract was carried out according to the process of Yotake and Sera [1912]. Hydrolysis was effected by sulphuric acid, and phosphotungstic acid used to precipitate amino compounds. Owing to a slight solubility of certain of these compounds in phosphotungstic acid, both precipitate and filtrate were examined. Three amorphous products were obtained (as described in the experimental part). These were optically active and contained both carbohydrate and amino groups. From the filtrate a compound was isolated which showed reducing properties, but on hydrolysis the power of reduction practically disappeared. The remaining two products showed no sign of reduction either before or after hydrolysis. The examination thus gave no trace of glucosamine glucosides or of complexes containing glucosamine. The substances actually isolated appear to be condensation compounds of sugars with amino acids.

The absence of glucosamine from these compounds indicates that in *Boletus edulis* the amino sugar does not occur in the form of an amino glucoside, as otherwise glucosamine would necessarily have been obtained by these methods.

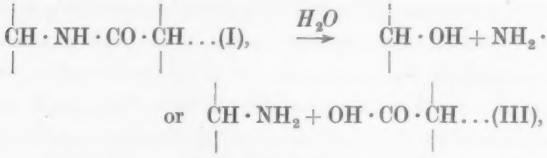
There remained, however, the possibility that the extraction of the carbohydrate fraction of *Boletus edulis* by means of sulphuric acid had been incomplete, and accordingly the undissolved residue was further examined. It was treated with concentrated hydrochloric acid, and kept just below boiling point for seven or eight hours. The insoluble material was filtered, and the solution concentrated and examined for glucosamine. Again no trace of this compound was found.

As however Reuter [1912] had obtained a definite amount of glucosamine from the carbohydrate fraction of *Boletus*, another hydrolysis was carried out on the original material, using on this occasion concentrated hydrochloric acid. The result confirmed Reuter's experience, as glucosamine hydrochloride was isolated in amount practically identical with that obtained by him.

These observations seem on first inspection contradictory, but they indicate that the production of glucosamine from *Boletus edulis* must be due to the formation of the amino sugar during the process of hydrolysis with concentrated hydrochloric acid. Hydrolysis with sulphuric acid yields a solution and a residue, neither of which contains glucosamine compounds. Since it is known that the action of concentrated sulphuric acid on chitin gives various results, in some cases entirely removing the amino group as ammonia, the absence of glucosamine in the products isolated might be accounted for in that way.

The work of Yotake and Sera [1912] however makes this seem improbable, as they isolated amino glucosides from reactions in which concentrated sulphuric acid was used. The conclusion arrived at therefore, is, that the glucosamine isolated from *Boletus edulis* under certain conditions is derived from a complex, of the nature of a glucoprotein, containing carbohydrate and amino acid residues, but does not exist in the form of a glucosamine glucoside.

This idea may be formulated as follows:



$$\text{CH}\cdot\text{NH}\cdot\text{CO}\cdot\text{CH}\ldots\text{(I)}, \xrightarrow{H_2O} \text{CH}\cdot\text{OH} + \text{NH}_2\cdot\text{CO}\cdot\text{CH}\ldots\text{(II)},$$

$$\text{or}\quad \text{CH}\cdot\text{NH}_2 + \text{OH}\cdot\text{CO}\cdot\text{CH}\ldots\text{(III)},$$

where (I) represents one characteristic grouping in a glucoprotein, which hydrolyses in two alternative ways, according to the hydrolyst used, giving rise to cleavage products (II) (a sugar and an acid amide); or to the products (III) (an amino sugar and an acid). Subsequent hydrolytic action on products (II) would yield ammonia and a carboxylic acid.

Experimental.

The *Boletus edulis* was supplied specially for the purpose of this research by Messrs Caesar and Loretz, of Halle, and the extraction of the material was carried out by Burroughs, Wellcome & Co., according to Reuter's method [1912]. The total weight received by them was 3565 grams, but after drying and grinding this was reduced to 3266 grams. The different extracts were:

(*a*)	Ether giving	72·3 grams
(*b*)	Alcohol ,, ...	1061·2 grams
(*c*)	Water ,, ...	785·3 grams

From aqueous washings 71·5 grams were obtained. The remaining undissolved matter weighed 1040 grams.

The material used in the research was the dried powdered residue left after extraction with water. As the method employed involved the manipulation of solutions of very large bulk, experiments were carried out on the scale described below, and since the products isolated were not fully examined and therefore cannot be definitely named, a system of lettering has been adopted to avoid confusion.

70 grams of the dried residue were ground in a mortar with 70 cc. of concentrated sulphuric acid. A thick paste was formed to which 350 cc. of water were added. The mixture was transferred to a large flask and boiled under a reflux condenser for six hours. The deep brown solution was filtered from the undissolved residue containing humic acid salts; the latter was again extracted with dilute sulphuric acid, boiled for some time, and the liquid filtered, leaving the undissolved *Residue A*.

The joint filtrates were then partially neutralised by the addition of a cold saturated solution of barium hydroxide, in order to remove 95 per cent. of the sulphuric acid present. The precipitate was filtered and the brown filtrate decolourised by boiling with charcoal. After being allowed to stand for forty-eight hours the solution was again filtered and 10 per cent. phosphotungstic acid solution added until precipitation was complete. From this, *Precipitate B* and *Filtrate B* were obtained. Before precipitation with phosphotungstic acid, polarimetric examination of the clear solution showed that it possessed a slight but perceptible dextrorotation, and, after precipitation, *Filtrate B* still showed optical activity.

Examination of Precipitate B. The light brown precipitate of phosphotungstates was washed by continued decantation with 5 per cent. sulphuric acid, filtered, rubbed with dilute sulphuric acid and again filtered. Finally, it was dissolved in dilute acid, leaving a dark residue (*Residue D*) which was filtered, and the clear solution was then treated with barium hydroxide until only a slight acidity remained. Barium sulphate was removed by filtering, the solution was saturated with carbon dioxide, and again filtered. In order to remove the last traces of barium, dilute sulphuric acid was cautiously added until a very slight acid reaction was produced. The clear solution was concentrated on the water bath at 30°–40° under diminished pressure, and when reduced to very small bulk, was further concentrated in vacuum until a thick syrup remained. On the addition of absolute alcohol, a white

precipitate was obtained (*Precipitate C*), which was filtered, washed with absolute alcohol, and dried.

Examination of Precipitate C. The product obtained was an amorphous yellow powder, deliquescent, insoluble in alcohol and ether. The melting point was somewhat indefinite (46°–50°), and the compound showed no action on Fehling's solution either before or after hydrolysis. Nitrogen was present in the form of amino nitrogen as indicated by the reaction with nitrous acid. Traces of carbohydrate were also present as shown by Molisch's test. In aqueous solution the compound showed a very slight rotation, $[\alpha]_D = +4{\cdot}1°$. As it was evident that this product contained no glucosamine residue, it was not further examined.

Examination of Residue D. The dark-brown undissolved material from recrystallisation of the phosphotungstic acid precipitate was dissolved in water, and treated in the same way as *Precipitate C*. It was similarly decomposed by barium hydroxide and, after filtering, the highly coloured filtrate was saturated with carbon dioxide, made slightly acid with dilute sulphuric acid, and concentrated in vacuum to a thick syrup. Precipitation with absolute alcohol gave a dark brown, amorphous, deliquescent powder. The product contained amino nitrogen, but showed no sign of reduction with Fehling's solution either before or after hydrolysis with concentrated hydrochloric acid, so that glucosamine complexes were absent. It gave no biuret reaction.

Examination of Filtrate B. The phosphotungstic acid filtrate was neutralised with barium hydroxide, the precipitate filtered and the solution concentrated on the water bath at 40° and finally in vacuum until a thick syrup was obtained. From this, on precipitation with absolute alcohol, a white product separated, which was filtered, washed with absolute alcohol and dried (*Precipitate E*).

Examination of Precipitate E. The substance was a white amorphous powder, soluble in water, insoluble in alcohol and ether. Ignition on platinum gave an inorganic residue consisting of barium salts. The compound contained amino nitrogen, readily reduced Fehling's solution, and in aqueous solution showed a slight rotation. Unsuccessful attempts were made to remove the inorganic matter. Part of it was precipitated as barium sulphate, but even after further purification barium salts still remained. The percentage of barium was therefore estimated and the product analysed.

Melting point 78°–80° (indefinite). The substance darkened slightly, and began to char at 110°.

Analysis of Purified Precipitate E.

Barium	12·43 %
Carbon	37·84 %
Hydrogen	6·97 %
Amino Nitrogen	2·325 %
Total Nitrogen	2·719 %
Rotation (in water) $[\alpha]_D$	− 13·3°

Hydrolysis with concentrated hydrochloric acid gave a yellow crystalline product which showed no reducing properties.

The high percentage of barium indicates that the compound is a salt, moreover the action on Fehling's solution observed before hydrolysis indicates that a carbohydrate group is present, and the fact that the total nitrogen is combined as amino nitrogen is important. It would thus appear that the compound is the barium salt of a carbohydrate acid combined with amino residues. These amino groups cannot however be attached to the penultimate position in the sugar chain, as otherwise glucosamine would have been produced on complete hydrolysis. This has already been shown from the results obtained by Irvine, Thomson and Garrett [1913], and by Fischer [1902].

Examination of undissolved Residue A. Since none of the products isolated showed any trace of the presence of glucosamine, *Residue A* was examined. It was shaken with concentrated hydrochloric acid for twelve hours, and then boiled gently under a reflux condenser for six hours. The undissolved material was filtered, and the dark-brown solution concentrated on the water bath under diminished pressure. No crystals of glucosamine hydrochloride separated, and precipitation with alcohol gave a slight dark-brown precipitate which showed no sign of reduction with Fehling's solution.

The barium sulphate precipitate obtained before precipitation with phosphotungstic acid was similarly extracted, and gave the same result.

As this process is the standard method for preparing glucosamine, it is evident that derivatives of the amino sugar could not be present.

Hydrolysis of the Original Extract with Concentrated Hydrochloric Acid.

50 grams of the original extract were treated with 600 cc. of concentrated hydrochloric acid, and boiled under a condenser for seven hours. The solution was diluted and filtered, and then concentrated on the water bath at 50°. A crystalline product separated, which was filtered, dried, and recrystallised

from water. It readily reduced Fehling's solution, and contained amino nitrogen.

Permanent rotation of the compound in water, $[\alpha]_D = +71{\cdot}5°$.
The amino nitrogen was estimated and found to be 7·567 %.
Glucosamine hydrochloride requires $[\alpha]_D = +72{\cdot}5°$; Amino nitrogen = 7·86 %.

The substance was undoubtedly glucosamine hydrochloride. The total yield of the recrystallised product was 1·7 grams which corresponds with the amount obtained by Reuter [1912] from a similar process of hydrolysis.

The author desires to express her indebtedness to Professor J. C. Irvine of St Andrews University for suggesting the topic of this research and for the interest he has taken in it; also to Dr W. N. Haworth for his supervision of the work.

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XXX. PRELIMINARY NOTE ON THE PREPARATION OF COLLODION DIALYSERS OF GRADED PERMEABILITY.

By WILLIAM BROWN.

From the Department of Plant Physiology and Pathology, Imperial College of Science and Technology, London.

(*Received July 6th, 1915.*)

In a recent publication, Walpole [1915] has described a method by which the permeability of collodion membranes is specified in terms of (1) the weight of dry nitro-cotton per sq. cm., (2) the "wetness" of the membrane.

In a series of experiments which have been in progress for some time, the author has found that collodion dialysers, constant in character and of a wide range of permeability, can be prepared by treating air-dried collodion membranes with varying percentages of alcohol in water. After such treatment for a suitable time, the membranes are thoroughly washed in water, and are then ready for use. The permeability of such membranes is directly related to the strength of the alcohol employed, so that by the use of various concentrations, membranes of almost any degree of permeability may be obtained: from those which will keep back e.g. copper sulphate, to those which are permeable to such a highly colloidal substance as aniline blue.

A second method of preparing graded dialysers which depends upon a process of differential drying has also been worked out. A full account of these researches will shortly be forthcoming.

REFERENCE.

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XXXI. A SIMPLE APPARATUS FOR FILTRATION UNDER DIMINISHED PRESSURE.

By JAMES COLQUHOUN IRVINE.

From the Chemical Research Laboratory, United College of St Salvator and St Leonard, University of St Andrews.

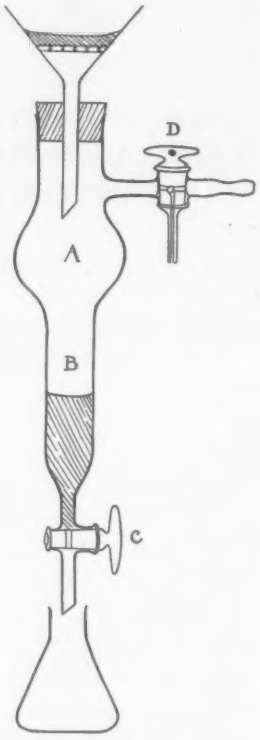
(*Received July 7th, 1915.*)

The separation of small quantities of crystalline matter from syrupy solutions, and, conversely, the recovery of residual syrups from semi-crystalline products, are common experimental difficulties. For work of this nature, the filtering tube shown below may be substituted with advantage for the ordinary filtering flask which, particularly when small quantities of material have to be manipulated, is inconvenient and involves undue experimental loss.

As the apparatus has not only served its original purpose well but has also found general application in filtrations under diminished pressure and in analytical work involving the Gooch crucible, a short note on its use may not be out of place, although it presents no very novel features.

The apparatus consists of a cylindrical tube furnished with a delivery stop-cock *C* at the lower end, the upper part being expanded into the bulb *A*. The side-limb carries a three-way stop-cock *D* and permits of connection to the pump. The filter funnel (or Gooch crucible) is attached as shown to the filter tube, which is supported by a clamp about *B*. When in use, *C* is closed and *D* opened to the pump and, on completing the filtration or when the tube is filled, the filtrate can be run off by admitting air to the apparatus by means of *D* and thereafter opening *C*.

Many advantages may be claimed for the arrangement. Syrupy liquids can be drained from the delivery tap without much loss and, as the apparatus can be enclosed in a hot-air bath, the filtration of viscous or saturated solutions is greatly facilitated. It is unnecessary while emptying the tube to remove the funnel or to detach the connection with the pump, which is kept running continuously. The objectionable practice (inevitable when a conical filtering flask is used) of pouring pure liquids out of the neck, into which the cork carrying the funnel is afterwards inserted, is avoided. When used in analytical work in conjunction with a Gooch crucible, the removal of washings for testing purposes is very conveniently done.



Practice with the apparatus reveals other and less obvious advantages, such as the facility with which the economical washing of a filtered residue by alternate slow filtration at ordinary pressure and rapid draining in a vacuum may be carried out, and also the ease with which the last traces of syrupy filtrates may be washed from the tube. This is best done by partially closing *D* to the pump and opening *C*. Air then enters by the delivery tap and bubbles rapidly through the solvent, thereby effecting thorough mixing and solution of the adhering syrup. A double purpose is served by the expansion *A*, as, in addition to adding to the capacity of the tube, it prevents liquids which boil under diminished pressure from rushing over into the side-limb, the bubbles being broken on rising to the bulb.

The diagram is drawn to scale ($\frac{1}{4}$ natural size) and gives the measurements which have been found most suitable for analytical work, or for filtrations of average scale. With the dimensions figured, 150 cc. of filtrate can be collected without emptying the tube, while the maximum and minimum sizes used allow of filtration of 300 cc. and 20 cc. respectively in each operation.

XXXII. THE RELATION OF SALIVARY TO GASTRIC DIGESTION.

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(*Received July 22nd, 1915.*)

Introduction.

The exact significance of the presence of ptyalin in the saliva has been a matter of dispute.

That an enzyme should be secreted in the mouth to be presently destroyed in the stomach, whilst a more efficient diastase is present in the secretion of the pancreas, is rather curious. The researches of Edkins on gastric secretin, however, show that dextrins may act as stimulants to the formation of this hormone. Since dextrin is one of the products of hydrolysis of starch resulting from the action of ptyalin, we can assign at least one valuable function to this enzyme. It occurred to me that another important function might on theoretical grounds be assigned to this ferment. Hedin [1906, 1912] has shown that charcoal and certain powders have the power of adsorbing enzymes, thereby hindering their activity. It is logical to suppose that colloidal starch solutions might adsorb pepsin and hence inhibit the activity of this enzyme. Saliva by hydrolysing the starch might prevent the adsorption of pepsin by the colloidal carbohydrate and would thus aid gastric digestion. The following experiments were conducted in order to elucidate this question.

Method.

Pepsin was allowed to act on fibrin in the presence of either colloidal starch solutions or starchy foods and the rate of digestion compared with that which occurred after previous hydrolysis of the carbohydrate by saliva

or takadiastase. The Grützner method was used to determine the rate of digestion, a solution of carmine serving as a standard colour against which the digestion tubes were compared. The tubes were placed in a shaker (to avoid errors due to diffusion) within a thermostat at 39° C.

In the various experiments pepsin was placed in contact with carbohydrate material for some time in order that adsorption might take place; this interval of time is indicated throughout the paper by the word *contact*.

The following solutions were prepared:

(1) A 2 per cent. starch solution.

(2) A 2 per cent. solution of takadiastase.

(3) 0·25 g. pepsin (Merck) dissolved in 100 cc. of 0·3 per cent. HCl.

The diastase solution was divided into two equal portions, one of which was boiled to destroy the ferment. Saliva was used in many experiments.

Exp. 1. *A.* 4 cc. unhydrolysed starch + 1 cc. pepsin HCl.

B. 4 cc. hydrolysed starch + 1 cc. pepsin HCl.

Contact 150 min. Equal quantities of fibrin were now added.

Fibrin digestion. *A* required 13 min.; *B* required 55 min.

There is evidently a marked difference in the activity of the pepsin after being in contact with colloidal starch.

In this experiment the contact was 150 min., which will be shown in the next experiment to be unnecessarily long.

Exp. 2. *Boiled Starch.*

A. 1 cc. pepsin HCl + 4 cc. hydrolysed starch.

B. 1 cc. pepsin HCl + 4 cc. unhydrolysed starch.

Unboiled Starch.

C. 1 cc. pepsin HCl + 4 cc. (starch + diastase).

D. 1 cc. pepsin HCl + 4 cc. (starch + boiled diastase).

Contact 20 min. Equal quantities of fibrin were then added.

Fibrin digestion. *A* required 13 min.; *B* required 60 min.; *C* required 13 min.; *D* required 13 min.

It is evident that:

(*a*) Adsorption of pepsin is very marked in 20 min.

(*b*) Unboiled, i.e. non-colloidal starch suspensions do not hinder peptic digestion.

Exp. 3. Soluble starch was prepared by treating pure potato starch with 7·5 per cent. HCl in thermostat at 40° until the resultant gave a clear solution

in warm water and a blue reaction with iodine. The acid was removed by washing with water to which alcohol had been added.

Erythrodextrin was not specially prepared for this experiment but a dextrin supplied by Merck, giving a rich red colour with iodine, was used. Saliva was substituted for takadiastase in all further experiments—except where otherwise indicated.

The carbohydrate solutions were all 2 per cent.

A. Unboiled starch suspension.
B. Unhydrolysed starch solution.
C. Hydrolysed starch solution.
D. Soluble starch.
E. Erythrodextrin.

3 cc. of each solution were taken and 1 cc. boiled filtered saliva added to each of tubes *A*, *B*, *D*, and *E*. 1 cc. unboiled saliva added to *C*. 1 cc. pepsin HCl added to *A*, *B*, *C*, *D*, *E*. Contact 30 min. Equal quantities of fibrin were then added.

Fibrin digestion. Time in min.

	A	*B*	*C*	*D*	*E*
I.	10	49	10	32	10
II.	10	50	10	35	14

It is evident from these results that soluble starch causes a certain amount of adsorption, but that the power is not retained by the erythrodextrin molecule. Unboiled starch, as in the previous experiment, did not exhibit any adsorptive effects.

Exp. 4. Further evidence as to the adsorption of pepsin by starch solutions is given by the following figures:

A. 1 cc. 0·25 per cent. pepsin solution + 3 cc. hydrolysed starch (2 per cent.).

B. 1 cc. 0·25 per cent. pepsin solution + 3 cc. unhydrolysed starch (2 per cent.).

Added equal quantities of fibrin to each. Contact 21 min. Removed fibrin and quickly washed with water. Added 5 cc. 0·3 per cent. HCl to *A* and to *B*.

Fibrin digestion. *A* required 15 min.; *B* required 46 min.

Another experiment showed that colloidal starch solution had little adsorptive effect upon HCl.

Exp. 5. A further test was performed to see if the presence of maltose might act as a catalyst to the action of pepsin HCl. Tubes were arranged as follows:

A. 4 cc. water (distilled).

B. 4 cc. 2 per cent. maltose.

C. 3 cc. water + 1 cc. saliva.

To each was added 1 cc. pepsin HCl. Contact 30 min. Equal quantities of fibrin were then added.

Fibrin digestion. *A* required 9 min.; *B* required 9 min.; *C* required 9 min.

Maltose has no catalytic effect upon pepsin HCl digestion and therefore cannot account for the difference between the rates of digestion of fibrin in the presence of unhydrolysed and hydrolysed starch.

Experimental work up to this juncture had been confined to pure starch solutions or the derivatives of starch. The experiments were next extended to certain food stuffs.

Exp. 6. *Maizena.* A preparation of maizena was made by adding the material to boiling water.

Tube *A.* 4 cc. maizena + 1 cc. boiled saliva.

Tube *B.* 4 cc. maizena + 1 cc. unboiled saliva.

Contact with saliva 60 min. *A* blue with iodine. *B* no colour with iodine.

1 cc. pepsin HCl added to each. Contact 20 min. Equal quantities fibrin then added.

Fibrin digestion. *A* required 30 min.; *B* required 12 min.

By the time that *A* had acquired the colour of the standard solution the fibrin in *B* had almost completely disappeared, although there was of course a considerable amount of fibrin still undigested in *A*.

Exp. 7. *Rice.* Boiled rice ground in a mortar. Equal weighed quantities placed in tubes *A* and *B*.

2 cc. boiled saliva added to *A*.

2 cc. unboiled saliva added to *B*.

Contact with saliva 60 min.

Added to each 1 cc. pepsin HCl. Contact 35 min. Fibrin was then added.

Fibrin digestion. *A* required 37 min.; *B* required 17 min.

Microscopical examination of material from tubes *A* and *B* after one hour's contact with saliva showed the following appearances:

A. Particles of comparatively massive appearance (as compared with those in *B*), staining darkly with iodine.

B. Particles of feathery disintegrated nature, giving a very slight stain or none whatever with iodine.

Exp. 8*a*. *Potato.* Potato boiled for three-quarters of an hour. Equal weighed quantities taken.

A. Potato + 2 cc. boiled saliva.

B. Potato + 2 cc. unboiled saliva.

Contact with saliva 45 min.

Added 1 cc. pepsin HCl to each. Contact 23 min.

Added 2 cc. 0·3 per cent. HCl to make total volume = 5 cc. and then added fibrin.

Fibrin digestion. *A* required 45 min.; *B* required 35 min.

Microscopical examination of iodine stained specimens of *A* and *B* showed that colloidal starch was still present within the cells of *A*, whereas it had been dissolved completely out of the cells of *B*.

Exp. 8*b*. The experiment was repeated using potato which had been boiled for two hours and had a very mashed appearance. Iodine stained specimens showed much cell debris. The cell contents had disappeared in many cases, leaving the skeleton of cellulose unstained.

Equal quantities *A* and *B* were taken as before and treated in exactly the same manner as in the previous experiment except that the contact with pepsin HCl was 20 min.

Fibrin digestion. *A* required 42 min.; *B* required 30 min.

It will be noted that potato exercises a comparatively small inhibitory effect upon peptic digestion.

This is probably accounted for by the fact that much of the colloidal starch still remains enclosed within the potato cells and hence does not exert an adsorptive effect upon pepsin.

Exp. 9. *Porridge.* Porridge ground in mortar to uniform consistency. Equal weighed quantities taken.

A. Porridge + 2 cc. boiled saliva.

B. Porridge + 2 cc. unboiled saliva.

Contact with saliva 120 min.

Added 1 cc. pepsin HCl. Contact 15 min.

Added 2 cc. 0·3 per cent. HCl to make total volume 5 cc., and then added fibrin.

Fibrin digestion. *A* required 29 min.; *B* required 10 min.

Treatment with iodine and microscopical examination showed that the starch had completely disappeared from *B*, whereas it was abundant in *A*. Disintegrated cells were present in both preparations.

Exp. 10. *Bread.* Bread was finely ground in a mortar. Equal weighed quantities taken and treated exactly as in Experiment 9, with the exception that salivary digestion occupied 70 min. and the contact with pepsin HCl was 30 min.

Fibrin digestion. *A* required 45 min.; *B* required 28 min.

Exp. 11. *Gum Acacia.* A 4 per cent. solution of gum acacia in water was made.

A. 3 cc. gum acacia + 1 cc. boiled saliva.

B. 3 cc. gum acacia + 1 cc. unboiled saliva.

Contact with saliva 75 min.

Added 1 cc. pepsin HCl. Contact 30 min. Fibrin was then added.

Fibrin digestion. After 65 min. there was equality in the liberation of colour in *A* and *B*, but both tubes were far below the standard tube in depth of colour.

The experiment was repeated with the following modifications:

A. 3 cc. gum acacia + 1 cc. saliva + 1 cc. pepsin HCl. Contact 35 min. Added fibrin.

B. 1 cc. pepsin at 39° for 35 min. Then added fibrin, followed immediately by 3 cc. gum acacia + 1 cc. saliva.

C. 1 cc. pepsin kept at 39° for 35 min. Then added 3 cc. gum acacia + 1 cc. saliva followed immediately by fibrin.

Fibrin digestion. After 90 min. there was equal liberation of colour in the three tubes, but still far from the standard colour tube. It appears that saliva does not alter the adsorptive power of gum acacia, and further that this adsorption is very complete and takes place almost instantaneously.

Exp. 12. *Biscuit.* A plain starchy biscuit was powdered in a mortar and equal quantities weighed out.

Microscopical examination of an iodine stained specimen revealed the fact that most of the starch grains were unburst. Equal quantities of taka-diastase (2 per cent. solution) boiled and unboiled respectively were added to tubes *A* and *B*. Contact with diastase 30 min.

No appreciable change—discernible to the eye—had taken place at the

end of this interval, and the enzyme was therefore allowed to act all night —4.25 p.m. to 9.30 a.m.

1 cc. pepsin HCl added to *A* and *B*. Contact 20 min. Fibrin was then added.

Fibrin digestion. *A* required 47 min.; *B* required 32 min.

Owing to the large amount of material undissolved which modified the colour of the liberated carmine, it was difficult to satisfy oneself as to the exact end point.

Starch grains were abundant in *A*, even after prolonged contact with diastase.

It is interesting to note in connection with the foregoing experiments that in the cat, dog, horse, sheep and ox the saliva is non-amylolytic. The last three animals consume large quantities of starchy food stuffs. The starch grains however are intact—unless the food has been artificially prepared by cooking, steaming, etc.—and do not form colloidal solutions and hence do not hinder gastric digestion.

Conclusions.

1. Peptic digestion is delayed in the presence of colloidal starch solutions through adsorption of enzyme, the time interval being increased four-fold in the presence of 2 per cent. starch solution.

2. There is a stage in the disruption of the starch molecule at which the phenomenon of adsorption of pepsin is lost. This occurs between the amylo-dextrin and erythro-dextrin stage.

3. Unboiled starch does not hinder the action of pepsin, a factor of importance in the dietetics of herbivora.

4. Cooked farinaceous foods—rice, potato, bread, porridge, maizena—all hinder peptic digestion if not first subjected to salivary digestion.

5. Gum acacia causes marked inhibition of peptic activity, and this is not prevented by previous contact with saliva.

6. The saliva of man, in virtue of its ptyalin, plays a considerable part in aiding gastric digestion by hydrolysing colloidal starch, which would otherwise adsorb pepsin.

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Hedin (1912), *Zeitsch. physiol. Chem.* **82**. 175.

XXXIII. THE REDUCING ENZYMES OF DRIED YEAST (LEBEDEFF) AND OF RABBIT MUSCLE.

By ARTHUR HARDEN and ROLAND VICTOR NORRIS.

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(*Received August 11th, 1915.*)

In a previous communication [Harden and Norris, 1914] it was shown that the power of reducing methylene blue is restored to dried yeast (Lebedeff), which has been washed until it has been rendered inactive in this respect, by a number of substances (e.g. salicylaldehyde) and the suggestion was made that these substances were capable of acting as acceptors for the oxygen activated during the reduction process. On the other hand many easily oxidisable substances (e.g. quinol) are without effect.

Further qualitative experiments carried out in a similar manner to those previously described have shown that comparatively few substances are able to act as acceptors in this way. The following list gives the results so far obtained, including those previously mentioned.

Positive.

Salicylaldehyde	Dihydroxyacetone	Bouillon
Benzaldehyde	Glycollic acid	Boiled yeast extract
Anisaldehyde	Citric acid	Normal horse serum
Isovaleraldehyde	Glyceric acid	Sterile milk
Phloroglucinol	Succinic acid	

Negative.

Quinol	Glucose	Creatine
p-Phenylene diamine	Laevulose	Asparagine
Pyrogallol	Glycocoll	Guanidine-acetic acid
Resorcinol	Alanine	Guanidine nitrate
Citral	Tyrosine	Glycol
Glycerol	Malic acid	Witte peptone
Propylene glycol	Tartaric acid	Acetone
2-3-Butylene glycol	Mandelic acid	Pyruvic acid
Formic acid	Yeast nucleic acid	Methylglyoxal
Acetaldehyde		

Of the hydroxy acids examined, lactic acid, either as free acid or sodium salt, was very active, whilst glycollic, glyceric and citric acid produced a very slow reduction. Sodium succinate also gave a very slow reduction.

Owing to their special interest the cases of acetaldehyde (negative) and lactic acid (positive) were more closely investigated.

Acetaldehyde. As acetaldehyde might be expected to exert some inhibitory action on the enzyme concerned in the reduction, special experiments were first made to ascertain how far this was the case.

Exp. 1. Four flasks were set up, each containing 5 cc. of a 10 per cent. suspension of the washed yeast (Lebedeff). To these were added the following solutions, and the mixtures incubated for two hours at 37°.

	Water cc.	Methylene Blue cc.	Boiled Yeast Washings cc.	1 per cent. Acetaldehyde cc.	Result
1.	6	1	0	0	—
2	1	1	5	0	Reduced in 49 min.
3.	5	1	0	1	—
4.	0	1	5	1	Reduced in 78 min.

In this experiment therefore the acetaldehyde has a pronounced inhibitory effect. When the concentration is halved however this inhibition ceases to be perceptible.

Exp. 2. A similar experiment was made with half the concentration of acetaldehyde.

	Water cc.	Methylene Blue cc.	Boiled Yeast Washings cc.	1 per cent. Acetaldehyde cc.	Result
1.	6·5	0·5	0	0	—
2.	1·5	0·5	5	0	Reduced in 21 min.
3.	6	0·5	0	0·5	—
4.	1	0·5	5	0·5	Reduced in 21 min

That the amount of aldehyde here employed is sufficient to reduce the methylene blue is proved by the fact that in presence of washed muscle (p. 335) half this amount produced complete reduction.

A further experiment with varying amounts of acetaldehyde also gave completely negative results.

Exp. 3. 10 cc. of a 10 per cent. suspension of washed yeast (Lebedeff) were used, together with the following additions.

	Saline cc.	Methylene Blue cc.	1 per cent. Acetaldehyde cc.	Result
1.	5	0·5	0	—
2.	3	0·5	2	—
3.	4	0·5	1	—
4.	4·5	0·5	0·5	—
5.	4·75	0·5	0·25	—
6.	4·9	0·5	0·1	—

Lactic Acid. Both free lactic acid and sodium lactate were found to have a positive action, and attempts were made to establish some quantitative relation between the amounts of methylene blue reduced and the amount of lactic acid oxidised and also to ascertain the nature of the products formed from the lactic acid.

1. No completely satisfactory solution of the former of these two problems was attained.

Any oxygen present in the various solutions interferes with this estimation by reoxidising the leuco-methylene blue and thus increasing the amount of lactic acid required for the reduction of a given weight of the colouring matter. Complete removal of the oxygen was very difficult to effect, especially from the yeast suspension, but the partial removal attained by boiling out the water and other solutions which were used and working in an atmosphere of nitrogen caused a great acceleration of reduction and a fall in the ratio of lactic acid to methylene blue.

Exp. 4. A solution of methylene blue was prepared containing 0·4 mg. per cc., i.e. 0·001 molar.

10 cc. of a 10 per cent. suspension of the washed yeast were taken in each case, along with 1 cc. N/100 lactic acid and 1 cc. of the methylene blue solution. The yeast suspension was made with water saturated with nitrogen.

(*a*) The solutions of methylene blue and lactic acid were boiled up and cooled in nitrogen, and the yeast suspension then added. Reduction occurred in 7 min.

(*b*) The solutions were boiled up and cooled in air and the yeast suspension then added. Reduction occurred in 21 min.

Exp. 5. Working with air free solutions in an atmosphere of nitrogen, the following results were obtained.

10 cc. of a 10 per cent. yeast suspension and 1 cc. 0·01 N lactic acid were used, the total volume being made to 16 cc. with water. Temperature 37°.

Methylene Blue cc.	Time for Reduction min.
2	19
3	43·5
4	68
5	90

The greatest amount of methylene blue reduced by 1 cc. of 0·01 N lactic acid was 5 cc., the molecular equivalent being 10 cc.

The interesting observation was made that the reduction in certain cases was greatly accelerated by exposure to light. This only occurred with solutions containing dissolved oxygen, and was ultimately traced to the accelerating influence of light on the reoxidation of leuco-methylene blue by oxygen, a reaction which is being further investigated.

2. *Products of oxidation of lactic acid.* The distillate from a mixture of the washed dried yeast, methylene blue and lactate gave a pronounced reaction for acetaldehyde. This suggested that the lactic acid was first converted by oxidation into pyruvic acid. The latter would then be decomposed by the carboxylase of the washed yeast into carbon dioxide and acetaldehyde, which, being incapable of further oxidation under the conditions of the experiment, could be detected in the liquid. Quantitative experiments showed that a considerable amount of acetaldehyde was produced. This amount however was considerably less than the equivalent of the lactic acid destroyed, and it is to be supposed either that a considerable proportion of the acetaldehyde condenses to alcohol or disappears in some other way, or that only a part of the lactic acid oxidised is converted into pyruvic acid. A special experiment showed that none of the aldehyde was converted into alcohol, there being somewhat more found in the absence than in the presence of methylene blue.

Pyruvic acid itself could not be detected.

Exp. 6. 20 g. of dried yeast were washed and made to 50 cc. and two mixtures made:

A. 25 cc. yeast suspension + 25 cc. sodium lactate solution (approx. N/5) + 0·4 g. methylene blue.

B. 25 cc. yeast suspension + 25 cc. sodium lactate solution.

These were incubated at 37° for 3·75 hours, at the end of which the methylene blue in *A* was completely reduced. The two mixtures were separately

distilled in steam to remove aldehyde, 55 cc. of distillate, cooled by ice and salt, being taken. To the residue oxalic acid was added, and the liquid then extracted with ether in a continuous apparatus, the ether distilled off, the residual mixture of oxalic and lactic acids boiled with calcium carbonate. The calcium oxalate and excess of carbonate were filtered off, washed and the calcium estimated in the filtrate and taken as equivalent to lactic acid.

The first distillate from *A* gave a strong Rimini reaction for acetaldehyde, whilst that from *B* was negative to this test.

The amount of acetaldehyde was in this case estimated by adding *p*-nitrophenylhydrazine dissolved in acetic acid and weighing the hydrazone, a control being carried out with water and the weight of precipitate subtracted.

Results.		
	Residual lactic acid in *A*	0·282 g.
	„ „ *B*	0·482 g.
	Loss of lactic acid	0·200 g.

Acetaldehyde nitrophenylhydrazone, *A*–*B*, 0·1111 g., corresponding to 0·0273 g. acetaldehyde.

This amount of acetaldehyde is molecularly equivalent to 0·0558 g. lactic acid. Hence in the reduction of 0·4 g. of methylene blue, 0·2 g. of lactic acid has disappeared and at least 0·0558 of this has been converted into acetaldehyde.

Exp. 7. A second experiment carried out with larger quantities gave similar results. In this case the aldehyde was estimated by the bisulphite method (treatment with bisulphite and titration of the excess by iodine). An aliquot portion of each distillate was tested for alcohol by boiling with silver oxide to remove aldehyde, distilling and estimating the alcohol by the method of Nicloux.

40 g. dried yeast were washed and made to 270 cc. 100 cc. of this suspension, 100 cc. of water and 50 cc. of a sodium lactate solution were taken in each case, 1·6 g. of methylene blue being added to *A*, but not to *B*.

Reduction occurred after 5·5 hours at 37°.

Results.		
	Residual lactic acid in *B*	1·235 g.
	„ „ „ *A*	0·415 g.
	Loss of lactic acid	0·820 g.
	Aldehyde produced in *A*	0·058 g.
	Lactic acid equivalent of aldehyde	0·118 g.
	Alcohol in *A*	0·025 g.
	„ „ *B*	0·098 g.

Hence in the reduction of 1·6 g. of methylene blue, 0·8 g. lactic acid has disappeared and at least 0·118 of this has been converted into acetaldehyde.

Preliminary experiments with rabbit muscle.

When the minced muscle of the rabbit is washed several times with saline, it behaves in a similar manner to dried yeast, and loses the power, possessed by the normal muscle, of reducing methylene blue.

The experiments with muscle are not however as satisfactory as those with yeast. When the muscle is very finely divided, the enyzme is removed as well as the acceptor. On the other hand, when the division is not so fine certain portions retain the acceptor and local reduction occurs. In any case the removal of the acceptor is not so complete as with dried yeast and reduction occurs in the controls in about 2–3 hours.

The chief interest in the results so far obtained with muscle lies in the fact that specific differences exist between the activating action of certain substances on the enzyme in muscle and on that in yeast. Thus acetaldehyde reactivates washed muscle, whereas, as shown above, it has no effect on washed yeast. Hitherto only qualitative experiments have been made with muscle and the positive results obtained are given below. The experiments are being continued.

Exp. 8. The muscle was minced and then washed with normal saline three times. Portions of 5 g. were then weighed out, a given volume of the substance to be tested and of the methylene blue solution, and if necessary of saline, added and the whole well shaken and incubated at 37°.

Substance tested	Vol. cc.	Methylene Blue cc.	Saline cc.	Time required for reduction at 37°, minutes		
Boiled muscle washings	5	0·5	—	14		
Boiled yeast washings	5	0·5	—	12·5		
Sodium lactate, 5 per cent.	1	1·0	8		28	
Acetaldehyde, 1 per cent.	1	0·5	9			41
,, ,,	0·25	0·5	9·75			46
Control	—	—	—	– in 50	– in 120	– in 60

None of the substances used reduced the methylene blue in the absence of the muscle.

SUMMARY.

1. The power of reducing methylene blue is restored to washed dried yeast (Lebedeff) by a number of oxidisable substances but not by others.

2. Lactic acid restores the power of reduction and is itself oxidised in the reaction, yielding acetaldehyde; the amount found however is not equivalent to the lactic acid lost. The lactic acid is probably first converted into pyruvic acid, which is then decomposed by the carboxylase of the yeast into acetaldehyde and carbon dioxide.

3. Rabbit muscle, like dried yeast, when washed loses the power of reducing methylene blue.

4. This power is restored to washed muscle by various substances. The enzyme concerned seems to be specifically different from that present in yeast, since acetaldehyde restores the power of reduction to washed muscle but not to washed dried yeast.

REFERENCE.

Harden and Norris (1914), *Biochem. J.* **8**, 100.

XXXIV. THE SUPPOSED SYNTHESIS OF URIC ACID FROM ITS DECOMPOSITION PRODUCTS BY TISSUE EXTRACTS.

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(*Received August 10th, 1915.*)

It has long been known that extracts of the organs of the lower animals possess the power of destroying uric acid [Stockvis, 1860; Brunton, 1905; Wiener, 1899, 1902, 1903]. Of the various decomposition products which have been reported at one time or another, allantoin has been shown by Wiechowski [1907] to be the real one, and this observer has demonstrated the quantitative conversion of the acid into this substance by the agency of an active ferment preparation from dog's liver. Ackroyd [1911] obtained the same result on perfusing surviving rabbit's liver with Ringer's solution containing dissolved sodium urate. Bezzola, Izar, and Preti [1909] found that uric acid dissolved in defibrinated ox blood was destroyed by perfusion through surviving dog's liver, and maintained that the uric acid was regenerated on saturating with carbon dioxide the solution containing the decomposition products and again passing it through the organ. Ascoli and Izar [1908] also demonstrated such a re-synthesis of uric acid from its decomposition products in the case of extracts of ox liver. When ox liver extract to which was added sodium urate was aërated and incubated, the uric acid was destroyed, but on saturating the resulting mixture with an inert gas such as carbon dioxide, hydrogen, or nitrogen, or by simply incubating the product in a closed vessel, the uric acid was quantitatively regenerated. Later, the same observers [1909] set out to determine what

substances could give rise to uric acid under these conditions. Having unsuccessfully tried several substances, allantoin among them, they found that dialuric acid and urea combined to form uric acid in presence of ox liver extract under anaërobic conditions.

The present paper is a repetition of their work. Their technique was employed in the preparation of the extract and during the incubation, but the uric acid was estimated by Hopkins' method, and not by the Ludwig-Salkowski silver method. The liver was obtained fresh from the slaughter-house, and was finely minced and digested for one hour with Ringer's solution or with 0·85 % sodium chloride solution in an incubator, followed by shaking for one hour. It was then strained through muslin. A solution containing a known weight of uric acid was added, together with 1 per cent. chloroform and 1 per cent. toluene, and the flask containing the mixture was placed in a thermostat at 37° and a current of air drawn through. At the end of the incubation, two equal volumes of the liquid were taken, and the uric acid determined in one of them at once. The second portion was then placed in a flask with more chloroform and toluene, stoppered, and again incubated, after which the uric acid was estimated. The proteins were coagulated in presence of dilute acetic acid in the heat, and the coagulum was filtered off, transferred back to the basin, boiled out with water and again filtered, the process being again repeated. To the filtrate and washings, ammonium chloride was added to the extent of 20–22 per cent. and 2 cc. per cent. of strong ammonia. The ammonium urate was decomposed with hydrochloric acid and titrated as in Hopkins' process.

The author was unable to show any difference in the uric acid content of the fluid before and after the anaërobic conditions, but found that the amount of uric acid in the latter case was equal to that which had not been oxidised by the uricolytic ferment in the extract. Therefore the results of Ascoli and Izar were not confirmed.

Experiment I. 500 g. of finely minced ox liver in 2000 cc. of Ringer's solution was maintained for one hour at 37°, and was then shaken for one hour. It was strained through muslin and 1 per cent. chloroform and 1 per cent. toluene added. A solution of 1·5608 g. of uric acid in 87 cc. of 0·1914 N lithium carbonate and water to 250 cc. were added and air was drawn through for 67 hours at 37°. Total volume was 2225 cc. after this period. Two portions *A* and *B* were then taken. *A* equalled 556 cc., *B* was 1124 cc. *A* was coagulated at once; *B* after anaërobic incubation at 37° for 114 hours with further addition of 1 per cent. chloroform and 1 per cent. toluene

was divided into two equal portions and the uric acid determined in one of them.

	Found	Calculated
A	Uric acid = 0·1957 g.	
B	Uric acid = 0·1824 g.	$\frac{1}{4} \times 1{\cdot}5608 = 0{\cdot}3902$ g.

Experiment II. 250 g. of dog's liver in 1000 cc. Ringer's solution was treated as before. 1·1882 g. of uric acid dissolved in 74 cc. of 0·1914 N lithium carbonate solution made up to 250 cc. were added. Air current for 45 hours at 40°. Final volume was 1230 cc. Two portions *A* and *B* were taken, each of 500 cc. *A* was coagulated at once, *B* after 72 hours of anaërobic incubation at 37° with a further portion of chloroform and toluene.

	Found	Calculated
A	Uric acid = 0·1282 g.	
B	Uric acid = 0·1050 g.	$\frac{500}{1230} \times 1{\cdot}1882 = 0{\cdot}4833$ g.

Experiment III. 347 g. of ox liver in 1500 cc. of Ringer's solution was treated as above. A solution of 1·0118 g. of uric acid in 60 cc. of 0·1914 N lithium carbonate solution and water to 250 cc. were added. Air current 42 hours at 37°. Final volume was 1800 cc. Two portions *A* and *B* were taken, each of 450 cc. *A* was coagulated at once, *B* after 72 hours of anaërobic incubation at 37° with a further addition of chloroform and toluene.

	Found	Calculated
A	Uric acid = 0·0791 g.	
B	Uric acid = 0·0435 g.	$\frac{1}{4} \times 1{\cdot}0118$ g. = 0·2529 g.

Experiment IV. 350 g. of ox liver in 1750 cc. of 0·85 per cent. sodium chloride solution was treated in the same way. A solution of 1·8155 g. of Merck's monobasic sodium urate (= 1·4120 g. of uric acid) in 240 cc. water and 10 cc. N NaOH solution were added, and air drawn through for 41 hours at 37°; a further 15 cc. of N NaOH was added after 19 hours as the reaction was acid. Two portions *A* and *B* were taken, each equal to a fourth of the mixture. *A* was coagulated at once, *B* after 46 hours of anaërobic incubation at 37° with a further addition of chloroform and toluene.

	Found	Calculated
A	Uric acid = 0·1326 g.	
B	Uric acid = 0·1426 g.	$\frac{1}{4} \times 1{\cdot}4120 = 0{\cdot}3530$ g.

Experiment V. 330 g. of ox liver in 1500 cc. of 0·85 per cent. sodium chloride solution was treated as usual. 25 cc. of N NaOH and a solution of 1·6013 g. of sodium urate (= 1·2454 g. of uric acid) in 500 cc. of water were added. Air stream for 42 hours at 37°. Two portions *A* and *B* were

taken, each equal to one-fourth of the total. *A* was coagulated at once, *B* after 27·5 hours of anaërobic incubation at 37° with further addition of chloroform and toluene.

	Found	Calculated
A	Uric acid = 0·0498 g.	
B	Uric acid = 0·0530 g.	$\frac{1}{4} \times 1{\cdot}2454 = 0{\cdot}3114$ g.

In the attempted synthesis of uric acid from dialuric acid and urea, two Erlenmeyer flasks with ground-glass stoppers were partially filled with equal volumes of liver extract. In one of them was placed a solution containing 2·0 g. of dialuric acid and 0·5 g. of urea dissolved in 0·85 per cent. sodium chloride solution. In the other, an equal volume of 0·85 per cent. sodium chloride solution was placed. To the contents of each flask 1 per cent. chloroform and 1 per cent. toluene were added. The flasks were thus filled to within 25 cc. of their total volume. They were stoppered and incubated for some days at 37°. The flasks were vigorously shaken twice a day. After the incubation, the contents were coagulated, and an endeavour was made to estimate the uric acid. This, however, was not found, as the following experiments show.

Experiment VI. 250 g. of ox liver and 1000 cc. of 0·85 per cent. sodium chloride solution as before. In flask *A* were placed 500 cc. of the extract together with 75 cc. of 0·85 per cent. sodium chloride solution and 1 per cent. of chloroform and toluene. In flask *B* were placed 500 cc. of the extract and a solution of 2·0 g. of dialuric acid and 0·5 g. of urea in 75 cc. of 0·85 per cent. sodium chloride solution, and 1 per cent. of chloroform and toluene. Incubation period was 220 hours at 37°. The contents were then coagulated.

A No precipitate with ammonium chloride and ammonia.

B No precipitate with ammonium chloride and ammonia.

Experiment VII. Similar to last, but incubation period was 92 hours.

A Very slight precipitate of ammonium urate.

B No precipitate of ammonium urate.

Experiment VIII. Similar to the above, but the mixture was made with 180 g. of ox liver and 1000 cc. of 0·85 per cent. sodium chloride solution, and the incubation was for 70 hours. Again no precipitate was obtained in either case.

I am unable to explain my failure to repeat the work of the Italian investigators. Their technique, so far as their published statements allow, was adopted in all essential details except in the method of estimating uric acid.

That the ammonium chloride method is reliable for the estimation of uric acid in tissue extracts is shown by the following observations upon human liver, which, as Walter Jones and his co-workers have shown, contains no uricolytic ferment.

Experiment IX. To each of two mixtures, *A* and *B*, containing 250 g. human liver in 250 cc. Ringer's solution, a solution of 0·6050 g. of uric acid in sodium carbonate and 250 cc. Ringer's solution were added.

A Coagulated at once gave 0·6055 g. of uric acid by the ammonium chloride process.

B Coagulated after six hours' aëration gave 0·6075 g.

Experiment X. A similar experiment, but in each case a solution of 0·7050 g. of uric acid in sodium carbonate and Ringer's solution to 250 cc. were added.

A Coagulated at once gave 0·6995 g. of uric acid.

B Coagulated after six hours' aëration gave 0·6980 g.

Summary.

The experiments of Ascoli and Izar were repeated, but no uric acid could be shown to have been formed:

(1) From the decomposition products of uric acid under the influence of ox liver extracts under anaërobic conditions;

(2) From dialuric acid and urea under the influence of ox liver extracts with exclusion of air.

I wish to express my sincere thanks to Professor Hopkins for suggesting this research and for his kind interest and advice.

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XXXV. THE PREPARATION AND COMPOSITION OF CASEINOGEN.

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Definition. In this paper the word *caseinogen* denotes the main protein present in fresh milk; *acidic caseinogen* the protein precipitated from milk by acid; and *casein* the protein precipitated from milk by the action of proteolytic ferments and calcium salts. The experimental facts upon which this terminology is based are set forward in the succeeding pages.

Introduction. The experiments described in this paper were made on caseinogen prepared directly from fresh milk by alcohol precipitation. A considerable quantity of work has been done on the protein precipitated from milk by the addition of acid to it (acidic caseinogen) on the assumption that this substance is the basic protein of milk. The prevalent hypothesis as to the relation of caseinogen to acidic caseinogen is founded upon an experiment of Ringer [1890] in which he showed that when a suspension of acidic caseinogen in water is triturated with calcium carbonate, the protein is dissolved forming a milk white solution. This experimental result is explained on the assumption that acidic caseinogen is an acid which reacts with calcium carbonate forming calcium caseinogenate (assumed to be identical with the caseinogen of milk) and liberating carbon dioxide thus:

$$\text{Acidic caseinogen} + CaCO_3 = \underset{\text{(Calcium caseinogenate)}}{\text{Caseinogen}} + CO_2.$$

The precipitation of acidic caseinogen from milk by the addition of small quantities of acid is expressed by the equation

$$\underset{\text{(Ca caseinogenate)}}{\text{Caseinogen}} + 2H\,\overline{A} = Ca\,\overline{A}_2 + \text{Acidic caseinogen}.$$

In the following pages evidence is brought forward in favour of the view that the main protein of milk consists of a complex of protein and calcium phosphate in definite proportions, and that the protein precipitated from milk by the addition of acid (acidic caseinogen) is different from that contained in the caseinogen complex. My conclusions are at variance with those

of other workers in so far as they have assumed that acidic caseinogen is essentially the same as caseinogen and have used the former protein as the basis of their experimental work. I propose to defer the consideration of the properties of caseinogen and the relation of acidic caseinogen to it.

The preparation of caseinogen from milk. In a previous paper [1907] I have shown that the precipitation of a protein from solution by alcohol usually involves two distinct processes; (i) precipitation of the protein; (ii) coagulation of the protein, this latter process rendering the precipitate insoluble in the original solvent. In the case of the protein of horse serum these two processes can be readily dissociated by precipitation above or below 14° C. Below this temperature, alcohol, added to the extent of 60 %, precipitates the protein in an unchanged condition; above this temperature the protein is not only precipitated but also rendered insoluble, the degree of insolubility of the precipitated protein being a function of (*a*) the temperature of precipitation, (*b*) the amount of alcohol used and (*c*) the length of time during which the precipitated protein is left in contact with the alcohol. These actions of alcohol are dependent upon the concentration of electrolytes contained in the protein solution, the less the ionic energy per unit volume the more effective the precipitating action of a definite quantity of alcohol, and the less insoluble the resulting precipitated protein. Similar facts hold good for the protein of milk. If alcohol, to the extent of 50 %, be added to fresh milk, caseinogen is not only precipitated but also coagulated. If, however, alcohol be added to the extent of 75 % to milk cooled below 15° C., the resultant protein precipitate, when freed from alcohol, is readily soluble in water.

The following method was used for the precipitation of caseinogen from milk by alcohol.

To one volume of separated milk three volumes of alcohol were added, both the milk and the alcohol being cooled below 15° C. The precipitate, after being strained through muslin and so freed from excess of alcohol, was ground up in a mortar with ether, filtered, and again extracted with ether until freed from fat and pigment. The final product was pressed between folds of filter paper and allowed to dry in the air. It is convenient to use fresh separated milk since milk fat accompanies caseinogen in the alcohol precipitation, and a large quantity of fat requires a large quantity of ether for its extraction. Also it is not advisable to cool the alcohol to 0° C. since at this temperature a large quantity of lactose is precipitated with the caseinogen.

The precipitate obtained from milk by alcohol consists of caseinogen admixed with small quantities of lactose, lactalbumin, and inorganic salts. The solid is not dry in the sense that it does not lose weight when heated to 100° C. It is advisable to keep it in an impure condition in a stoppered bottle since if heated to 100° C. in an air bath it passes into a modification insoluble in water.

The composition of caseinogen. The composition of caseinogen was determined by making a series of experiments on the impure substance. The caseinogen was not separated from all impurities before determining its properties since such a procedure involves prolonged manipulation, and caseinogen being a labile protein readily passes into modifications which differ from the original substance. The crude substance when suspended in water at 37° C. forms a milk white solution. This solution takes place slowly (about thirty minutes) probably owing to the difficulty of the water getting into intimate contact with the large protein aggregates. An important fact to notice, however, is that the crude substance contains no substances insoluble in water such as calcium phosphate, or a protein comparable in solubility to acidic caseinogen.

The impurities associated with caseinogen in the crude alcoholic precipitate can be estimated by taking advantage of their solubilities compared with that of caseinogen. Caseinogen is practically insoluble in water at 15° C., whilst lactose and lactalbumin are freely soluble at this temperature. Therefore in order to determine the amount of caseinogen contained in five grams of the solid, this quantity of substance was suspended in water, made up to 100 cc., and allowed to extract at room temperature (15° C.) for twenty hours. At the end of this time the suspension was filtered, and the weight of solid in a measured quantity of the filtrate determined by drying over a water bath and completing the drying in an air bath at 120° C. for four hours.

The following figures give the results of such an analysis for one gram of the impure solid:

Weight of substance soluble in water at 15° C. contained in 20 cc. of filtrate	0·19 g.
Loss in weight of 1 g. of solid after drying at 120° C. for four hours (H_2O, etc.) ...	0·202 g.
	0·392 g.

Therefore one gram of the solid contained 0·608 g. of caseinogen.

(*a*) *The calcium and phosphorus content of caseinogen.* The amount of calcium contained in caseinogen was determined by the method of Hurtley and Kahn [1915]. The method consists of ashing a known weight of caseinogen, dissolving the ash in a small quantity (5 cc.) of 25 % phosphoric acid, diluting to 100 cc. with water, precipitating the calcium by slowly adding from a burette 100 cc. of 5·524 % potassium oxalate, washing the precipitate with water till free from soluble oxalate, dissolving it in dilute sulphuric acid, and determining the quantity of freed oxalic acid (and consequently calcium) by titration with tenth normal permanganate. The following figures give the results of a typical experiment made in this way.

Calcium contained in one gram impure, undried caseinogen

$$= 12{\cdot}8 \text{ cc. } KMnO_4 \text{ (0·1 N)}.$$

The calcium contained in the substances soluble in water at 15° C. = 3·08 cc. $KMnO_4$ (0·1 N).

From the figures previously given the weight of caseinogen contained in one gram of impure substance = 0·608 g.

Therefore the amount of calcium contained in one gram of pure caseinogen = 15·84 cc. $KMnO_4$ (0·1 N) = 0·0317 g. calcium.

The amount of phosphorus contained in the caseinogen was determined by a modified Neumann's [1902] method. Amounts of impure caseinogen varying in weight from 0·2 g. to 0·5 g. were oxidised by the method described by Plimmer and Bayliss [1906]. In this oxidation it was necessary to use small quantities of the protein and prolonged heating with nitric acid to ensure complete destruction of the protein. The resultant fluid was diluted to 100 cc. and after heating to 80° C., 100 cc. of a phosphate precipitating mixture was added, and the heating continued on a water bath for 15 minutes. For precipitation of the phosphate a filtered mixture of ammonium nitrate and ammonium molybdate in nitric acid made up in the proportions recommended by Halliburton [1914] was used.

This solution does not deposit molybdic acid in the cold but when heated to 100° C. the white modification of molybdic acid separates out. The precipitation of molybdic acid is prevented by sulphuric acid, and it was essential therefore so to conduct the preliminary hydrolysis of the protein that sufficient sulphuric acid remained in the hydrolysed fluid to prevent the precipitation of molybdic acid, but not that of the ammonium phosphomolybdate, after the addition of the precipitating mixture, and subsequent heating on a water bath. The following gives the experimental

results obtained from the same sample of crude caseinogen as was used in the experiments above.

P_2O_5 in one gram impure caseinogen = 38·5 cc. NaOH (0·5 N).

P_2O_5 contained in the substances soluble in H_2O at 15° C. = 9 cc. NaOH (0·5 N).

Therefore the P_2O_5 contained in the caseinogen was equivalent to 29·5 cc. NaOH (0·5 N).

The weight of caseinogen contained in one gram of impure substance = 0·608 g.

Therefore the amount of P_2O_5 contained in one gram of pure caseinogen = 48·6 cc. NaOH (0·5 N) = 0·062 g. P_2O_5.

(*b*) *The calcium and phosphorus contents of acidic caseinogen.* On the addition of acid to a solution of caseinogen a protein (acidic caseinogen) is precipitated. A comparison of the calcium and phosphorus contents of caseinogen and acidic caseinogen yields interesting results. The acidic caseinogen used in these experiments was prepared by dissolving the impure caseinogen in water, precipitating the acidic caseinogen by the addition of an optimal quantity of acetic acid and washing the precipitate with water, alcohol and ether. It may be remarked that acidic caseinogen prepared in this way is identical in phosphorus content with that prepared by the acidic precipitation of fresh milk, and with that of Merck's "pure casein" (acidic caseinogen) prepared by Hammarsten's method. The identity of the results obtained with these specimens of acidic caseinogen affords presumptive evidence in favour of the view that caseinogen precipitated from milk by alcohol is the protein of milk in an unaltered condition.

Acidic caseinogen prepared from caseinogen contains no calcium and in this respect also agrees with the analysis of acidic caseinogen obtained from milk by Hammarsten's method. The absence of calcium is in marked contrast to the large quantities of this element contained in caseinogen.

The phosphorus content, however, although less than that of caseinogen, is large. The following figures give the average of a series of experiments.

P_2O_5 in one gram acidic caseinogen (pure) = 16·5 cc. NaOH (0·5 N) = 0·021 g. P_2O_5.

(*c*) *A comparison of the calcium and phosphorus contents of caseinogen and acidic caseinogen.* From the figures given above it may be seen that one gram of caseinogen contains 0·0317 g. calcium and 0·062 g. P_2O_5, and one gram of acidic caseinogen contains no calcium and 0·021 g. of P_2O_5.

The question arises as to whether these figures give any indication of

the relation of caseinogen to acidic caseinogen, and the manner in which calcium is contained in the caseinogen complex. We may legitimately assume that the phosphorus contained in acidic caseinogen is an organic combination since there are no inorganic bases to combine with it. If this amount of phosphorus be organically contained in caseinogen also, there is now 0·0406 g. P_2O_5 contained in one gram of caseinogen not accounted for. On the assumption that the calcium is associated with the residual P_2O_5 (0·0406 g.) in the caseinogen complex as $Ca_3(PO_4)_2$, 0·034 g. of calcium would be required. The approximation of the theoretical quantity of calcium required (0·034 g.) with that actually determined (0·0317 g.) offers strong evidence in support of the hypothesis that in caseinogen the phosphorus is present in two forms (*a*) in organic combination, (*b*) associated with calcium as calcium phosphate.

(*d*) *A comparison of the quantity of phosphorus contained in acidic caseinogen with that of inorganic phosphorus contained in caseinogen.* The amount of phosphorus contained in one gram of acidic caseinogen is 0·021 g. P_2O_5. On the assumption that a molecule of acidic caseinogen contains at least one atom of phosphorus, the least molecular weight for caseinogen is 3375, i.e. 3375 g. of acidic caseinogen contain one gram atom of phosphorus.

The amount of inorganic phosphorus assumed to be present in one gram of caseinogen is 0·0406 g. P_2O_5, the assumption being that this phosphorus is present in the protein complex associated with calcium in the same proportions as in calcium phosphate. From these figures it may be calculated that 142 g. of P_2O_5 (the quantity contained in a gram molecule of calcium phosphate) are contained in 3480 g. of caseinogen. The remarkable approximation of the two numbers giving (1) the least molecular weight of acidic caseinogen on the assumption that an atom of phosphorus is contained in the molecule (3375) with (2) the quantity of caseinogen complex which contains a gram molecule of calcium phosphate (3480 g.) affords strong evidence in favour of the assumption made above, i.e. that caseinogen contains phosphorus in two forms (*a*) organically combined in the protein molecule, identical with the phosphorus in acidic caseinogen (0·021 g. P_2O_5 per gram of caseinogen) and (*b*) contained in caseinogen complex in association with calcium as $Ca_3(PO_4)_2$ (0·406 g. P_2O_5 per gram of caseinogen). This hypothesis receives further confirmation from a consideration of the quantity of acetic acid required to precipitate acidic caseinogen from caseinogen.

(*e*) *The precipitation of caseinogen by acetic acid.* On the addition of acid to a solution of caseinogen no precipitate is produced until a definite

quantity of acid has been added and then the whole of the acidic caseinogen comes out of solution. The following figures illustrate this statement.

Caseinogen solution	H_2O	Acetic acid (0·1 N)	
1 cc.	8·9 cc.	0·1 cc.	no pp.
	8·8 „	0·2 „	„
	8·7 „	0·3 „	„
	8·6 „	0·4 „	„
	8·575 cc.	0·425 cc.	trace pp.
	8·55 „	0·45 „	complete pp.

This precipitation will be considered more fully later. At present it may be noted that a definite quantity of acid precipitates a definite quantity of caseinogen, and that these relations are almost independent of temperature or dilution. In the above experiment the quantity of caseinogen contained in the solution was 2·68 g. per 100 cc. Therefore 0·45 cc. acetic acid (0·1 N) precipitated 0·0268 g. caseinogen, or in ordinary terminology, a gram molecule of acetic acid precipitated 583 g. of caseinogen from solution as acidic caseinogen. Now six molecules of acetic acid are equivalent to one molecule of calcium phosphate $Ca_3(PO_4)_2$. Therefore an amount of acetic acid which is equivalent to a gram molecule of calcium phosphate precipitates 3498 g. of caseinogen from solution as acidic caseinogen. The interest of this number lies in the fact that the quantity of caseinogen which contains a gram molecule of calcium phosphate is 3480 g.

Discussion of results.

The above results may be stated shortly as follows:

I. On the assumption that one unit of acidic caseinogen contains one atom of phosphorus the weight of acidic caseinogen containing one gram atom of phosphorus is 3375 g.

II. On the additional assumption that the phosphorus of caseinogen is contained in two forms (i) as organic phosphorus equal in quantity to that contained in acidic caseinogen, (ii) as inorganic phosphorus being contained in the protein complex as calcium phosphate we find (*a*) that the quantity of calcium and inorganic phosphorus contained in caseinogen bears the same relation to one another as is expressed in the formula $Ca_3(PO_4)_2$ and (*b*) that the amount of caseinogen which contains one gram molecule of calcium phosphate is 3480 g.

III. The amount of caseinogen precipitated by six gram molecules of acetic acid, i.e. a quantity equivalent to a gram molecule of calcium phosphate, is 3489 g.

The approximation of the above numbers—the quantity of acidic caseinogen containing a gram atom of phosphorus (3375 g.); the quantity of caseinogen containing a gram molecule of calcium phosphate (3480 g.); and the quantity of caseinogen precipitated by six gram molecules of acetic acid (3489 g.)—offers strong evidence in favour of the assumptions made and justifies the conclusions that,

(1) a unit of acidic caseinogen containing one atom of phosphorus has a weight of approximately 3500;

(2) caseinogen consists of a complex of protein having the same weight as the acidic caseinogen unit associated with one molecule of calcium phosphate;

(3) the precipitation of acidic caseinogen from caseinogen by acetic acid involves the reaction of the added acid with equivalent quantities of calcium phosphate, one molecule of calcium phosphate being replaced by six molecules of acetic acid.

The following tentative hypothesis is put forward as to the constitution of the caseinogen complex.

The protein contained in the complex may be expressed by the formula

$$R\begin{cases}(NH_2)_2\\(X)\end{cases}$$

of which the radicle (X) has acidic properties, possibly due to the phosphorus contained in it. The acidic radicle (X) reacts with the $Ca_3(PO_4)_2$, forming X Ca, whilst the liberated acid calcium phosphate combines with the amino group, the resultant complex having the formula

$$R\begin{cases}(NH_2)_2\,Ca_2H_2(PO_4)_2\\X\ Ca\end{cases}$$

The experimental evidence in favour of this decomposition of $Ca_3(PO_4)_2$ into $Ca(OH)_2$ and $Ca_2H_2(PO_4)_2$ is derived from the work of Cameron and Seidell [1904] who showed that tricalcium phosphate and monocalcium phosphate are considerably decomposed by water but that dicalcium phosphate is relatively stable in this medium.

Summary.

(1) Caseinogen may be prepared from milk by alcohol precipitation. The protein forms a milk white solution in water possessing all the properties of caseinogen contained in milk.

(2) Comparative analyses of caseinogen and acidic caseinogen indicate that caseinogen is composed of a complex of protein and calcium phosphate,

approximately 3500 g. of caseinogen containing a gram molecular weight of tricalcium phosphate.

(3) The amount of phosphorus contained in acidic caseinogen shows that a gram atom of phosphorus is contained in approximately 3400 g. of acidic caseinogen.

(4) Six gram molecules of acetic acid (a quantity equivalent to one gram molecule of calcium phosphate) precipitate acidic caseinogen from approximately 3500 g. of caseinogen.

(5) These relations suggest that the protein unit of acidic caseinogen has a weight of approximately 3400 g.; that caseinogen consists of a complex containing one unit of protein and a molecule of tricalcium phosphate; and that the precipitation of acidic caseinogen from caseinogen by acetic acid may be expressed thus:

$$\underset{\text{(caseinogen)}}{\text{Protein, } Ca_3(PO_4)_2} + 6\,H\bar{A} = \underset{\text{(acidic caseinogen)}}{\text{Protein, } 6\,H\bar{A}} + Ca_3(PO_4)_2$$

The following relation of the protein to the calcium phosphate in caseinogen has been suggested:

$$R\begin{cases}(NH_2)_2\,Ca_2H_2\,(PO_4)_2\\ X\ Ca\end{cases}$$

where the group (X) has feeble acidic properties.

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XXXVI. THE COMPOSITION OF "LECITHIN," TOGETHER WITH OBSERVATIONS ON THE DISTRIBUTION OF PHOSPHATIDES IN THE TISSUES AND METHODS FOR THEIR EXTRACTION AND PURIFICATION.

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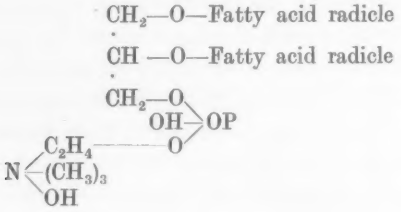
About the year 1846 Gobley succeeded in obtaining from egg yolk and other organs a substance of a fat-like nature containing nitrogen and phosphorus which he called lecithin [1846]. This substance was soluble in alcohol and in ether, formed an emulsion when rubbed up with water and yielded fatty acids and glycerophosphoric acid on hydrolysis. These observations of Gobley furnished a basis for the structure of the lecithin molecule, which appeared to be completed by the discovery of Liebreich [1865] and Strecker [1868] that the base choline was also obtained as a product of lecithin hydrolysis.

Lecithin was therefore defined as an ester compound of glycerophosphoric acid substituted by two fatty acid radicles with a base, choline, according to the following formula which is generally accepted.



Later it was found that other somewhat similar bodies were present in different organs and tissues; for this general class of substances the name *phosphatides* was suggested by Thudichum [1884].

Lecithin is soluble in all the ordinary organic solvents with the exception of acetone. It differs from a substance known as kephalin in that the latter is insoluble in alcohol. So far, as has already been pointed out, the only phosphatides identified with certainty as distinct substances are lecithin, kephalin, cuorin and sphingomyelin.

A short description of these substances and their probable distribution in the tissues will help to render clear certain problems discussed later.

Both kephalin and lecithin are monamino-monophosphatides (N : P = 1 : 1). Cuorin on the other hand is a monamino-diphosphatide (N : P = 1 : 2), while sphingomyelin is a diamino-monophosphatide (N : P = 2 : 1). The first three of these substances are more or less of a plastic consistency and very hygroscopic, while they are so labile that exposure to air and light for a comparatively short time considerably modifies certain of their properties. They do not crystallise and on account of their liability to oxidation must be preserved in evacuated desiccators. Sphingomyelin on the other hand is a pure white crystalline substance and has physically no resemblance to the other phosphatides mentioned. It is practically non-hygroscopic and does not oxidise on exposure to air.

While these substances have many properties in common, their separation and identification depend chiefly on the following points.

	Lecithin	Kephalin	Cuorin	Sphingomyelin
(1)	Soluble in ether and in alcohol	Sol. in ether Insol. in alcohol	Sol. in ether Insol. in alcohol	Insol. in ether Sol. in hot alcohol Insol. in cold alcohol
(2)	Insol. in acetone	Insol. in acetone	Insol. in acetone	Insol. in acetone
(3)	N : P = 1 : 1	N : P = 1 : 1	N : P = 1 : 2	N : P = 2 : 1

The two chief solvents in common use for extracting phosphatides from tissues are ether and alcohol. Ether extracts all four phosphatides to some extent, for although sphingomyelin is insoluble in ether it is fairly soluble in an ether extract of tissue. On the other hand, only a small part of the total tissue phosphatides are capable of being extracted by means of ether. After extracting with ether to a point at which only the merest traces of phosphatides can be obtained, it is found that subsequent extraction with alcohol results in the removal of further large amounts of these substances.

Attempts have been made to explain this phenomenon on the supposition that the ether fails to penetrate the tissues—that the difficulty is simply

a mechanical one. It has been pointed out that when fats or phosphatides are mixed with protein matter and the whole mass dried and then extracted with ether, only a fraction of the original ether-soluble material is removed. While this is true to a degree, it is equally certain that the fraction which can be removed bears a much higher proportion to the total fatty matter present than is the case in extraction of the tissues.

In extracting dried normal kidneys with ether not more than 15 % to 30 % of the total phosphatides can generally be obtained; often the amount is even less than this. On subsequent extraction with alcohol the remainder comes out with ease.

If, however, the kidney is one showing marked fatty degeneration, the greater part of the phosphatides is obtained by ether extraction.

From this it would appear that part of the phosphatide material at any rate is present in some kind of ether-insoluble combination with the proteins; this combination is acted on by alcohol and the phosphatide liberated; it is then easily extracted by ether in so far as physical difficulties permit. In this connexion an interesting observation has just been made by Osborne and Wakeman [1915]. In an investigation on milk they found that the phosphatides were present in the precipitated protein matter; on drying this protein and extracting it with ether no lecithin whatever was obtained, while subsequent treatment with alcohol removed a considerable amount. The explanation advanced by Erlandsen [1907] that the ether-extractable material was "lecithin" while the ether-soluble substance subsequently obtained by means of alcohol was not "lecithin" but a new substance—a diamino-monophosphatide—has been shown to be untenable [MacLean, 1913, 1].

By far the greater part of the tissue phosphatides generally consists of the so-called "lecithin" together with some kephalin. Cuorin appears to be present only in small amount, and from many tissues only a trace of sphingomyelin can be obtained.

On an alcohol-soluble nitrogenous impurity associated with "lecithin."

The observations already made show that alcohol is a more suitable solvent than ether for extracting lecithin from tissues. For the reasons given, alcohol is now generally used, the usual procedure being to dry the tissue as quickly as possible, grind it to a fine powder and extract thoroughly with various changes of the solvent, a shaking machine being used. On evaporating the

alcohol from the combined extracts the residue is taken up with ether, and the phosphatides precipitated by the addition of excess of acetone to the ethereal solution. By repeating the process several times all acetone-soluble bodies such as cholesterol and fatty acids are removed. The final residue is supposed to consist almost entirely of phosphatides and this is divided into "lecithin" and kephalin by fractionation with alcohol. The alcohol-insoluble part is considered as kephalin; the alcohol-soluble as "lecithin."

In every case, however, in which this method is used as a basis for the preparation of phosphatides, the resulting products are far from pure and the "lecithin" fraction often contains as much as 50 % of an extraneous product. This substance which has already been referred to [MacLean, 1912] is soluble in water and on treatment of the "lecithin" fraction by means of acetone and water [MacLean, 1912] it remains in the acetone-water solution. On evaporation of this solution the substance is obtained as a sticky syrupy mass which is insoluble in ether and in *absolute* alcohol but easily soluble in alcohol containing a trace of water. It is exceedingly soluble in water; on standing for some days an aqueous solution gradually deposits small round white crystals. These crystals are insoluble in cold water, but dissolve in boiling water to be reprecipitated on cooling. After recrystallising in this way several times, a substance or mixture of substances is obtained which has a very high nitrogen content and evidently belongs to the purine group.

In one case small round concentric white crystals were isolated which had all the properties of carnine. After drying at 105°, analysis showed that the substance contained 28·55 % nitrogen. On heating it changed colour about 230° and blackened at 240°. With silver nitrate a white flocculent precipitate was obtained which did not dissolve in ammonia or nitric acid. A precipitate was also given with basic lead acetate and with mercuric chloride, while neither neutral lead acetate nor mercuric nitrate gave any result. In its properties and nitrogen content this substance appears to be identical with or closely related to the base carnine found by Weidel in American meat extract. In another sample a small amount of a substance which appeared to be impure hypoxanthine was isolated: it contained about 40 % nitrogen. The mother liquor from which these substances separate is distinctly acid in reaction, and it is probable that these bodies are set free by a process of decomposition and are not present in the free state in the original liquid. This is suggested by their extreme insolubility in cold water and the difficulty experienced in redissolving them in the

mother liquor after separation has once taken place. The fact that complete separation takes days or even weeks is also suggestive in this connexion.

Owing to the high nitrogen percentage in these bodies it is obvious that a small quantity present in lecithin would materially influence the N : P ratio.

When the separation of these bodies is complete an absolutely clear straw coloured mother liquor is obtained which remains clear for a very long time.

On evaporating, a sticky hard gum-like substance remains. This substance is soluble in alcohol containing a trace of water and appears to dissolve in solutions of lecithin. Further, like phosphatides in general, *it is precipitated by acetone, by cadmium chloride, and also by platinum chloride.* The body appears to be of a very complex nature and so far it has been impossible to determine its chemical constitution. Different specimens contain on an average about 6 % of nitrogen, and there is generally a small amount of phosphorus also present, though the latter may not amount to more than a trace. When tested by Van Slyke's method a considerable part of its nitrogen was found to be present in the amino form. On hydrolysis some specimens yielded a very small amount of fatty acids, but this was probably due to contamination with lecithin, for a specimen has been obtained recently which, on hydrolysis with HCl, gave no trace of fatty acids. It decolourised Fehling's solution on heating but no precipitate occurred: on prolonging the boiling however a dense precipitate was formed.

Attempts to fractionate the water-soluble substance.

Some of the substance was rubbed up thoroughly with ether to remove any traces of fatty acids or phosphatides that might be present. The ether was decanted off and the residue dissolved in about 20 cc. water to which 30 cc. alcohol and 50 cc. ether were added. On the addition of the ether, part of the substance was precipitated as an oily liquid. This was dried and analysed. It contained 5·7 % nitrogen and 0·9 % phosphorus.

The part that remained soluble in the water-alcohol-ether mixture was obtained by evaporating off the solvent. It was of a brown colour and thick syrupy consistency. This was dissolved in water and excess of mercuric chloride added which produced an immediate precipitate.

After standing for two hours this precipitate was filtered off = *Precipitate A*. The filtrate was allowed to stand, and gradually deposited some more substance

which was filtered off after 24 hours = *Precipitate B*. Precipitate *A* was decomposed in water by means of sulphuretted hydrogen, the insoluble sulphide separated and the filtrate evaporated to dryness *in vacuo*. A crop of crystals was obtained; the nitrogen estimation gave the following figures:

0·0164 g. required 4 cc. N/10 H_2SO_4 = 34·1 % N.

In certain of its properties it agreed with creatinine, though on treatment with sodium nitroprusside and alkali, or picric acid and alkali, no red colour was produced. Sufficient of the substance was not obtained to identify it with certainty; it was probably not a pure substance. Precipitate *B* was treated in the same way as above, and a small amount of crystalline residue was obtained. Unlike the corresponding substance obtained from precipitate *A*, this substance gave marked creatinine reactions and presumably contained creatinine which might have been formed from creatine during the various manipulations employed.

Thus far there is evidence that the original water-soluble impurity contained carnine or somewhat similar bases, and a trace of creatinine, the latter perhaps being an artificial product. The filtrate *M* from the mercuric chloride precipitate was decomposed by sulphuretted hydrogen and the sulphide separated by filtration. The filtrate was evaporated to dryness on the water bath and the residue dissolved in alcohol. After filtration the alcohol was removed *in vacuo* and the residue dried and analysed for N and P. It was a brownish hard hygroscopic material.

0·0708 g. required 3·09 cc. N/10 H_2SO_4 = 6·11 % N.
0·2234 g. gave no trace of phosphorus.

These results sufficiently indicate the complex nature of this substance; the fact that it is so exceedingly difficult to separate from the phosphatides by the ordinary methods of purification is undoubtedly responsible for the appearance in the literature of many supposed new phosphatides.

In very many cases it is quite obvious from the methods employed by several investigators that the phosphatides obtained and supposed to be pure substances must have been contaminated with this impurity.

The phosphatides can be entirely separated from this impurity by emulsification with water and precipitation with acetone as already described in a former paper [MacLean, 1912].

Some anomalous results obtained in the extraction and preparation of phosphatides.

The simplest method for the extraction of the chief bulk of the phosphatides from the tissues is by means of alcohol, the dried tissue being thoroughly exhausted with this solvent. If the cuorin is also wanted it is necessary to use ether, as this substance is apparently quite insoluble in alcohol or in alcoholic solutions of phosphatides. When the alcohol filtrates are concentrated, the residue dissolved in ether and the phosphatides precipitated by excess of acetone, it is generally found that a good deal of the acetone precipitate is insoluble in absolute alcohol. This insoluble part on further purification is found to have a N : P ratio of 1 : 1 and constitutes the substance known as kephalin; the alcohol-soluble "lecithin" generally predominates in amount. If, however, the same kind of tissue is obtained in as fresh a condition as possible and carefully and quickly dried, either by immersing the finely divided substance for a few minutes in excess of alcohol or by exposing it in thin films to a current of air at 30°, it is often found that the alcoholic extract, when treated in the usual way with acetone, gives a precipitate which is entirely soluble in alcohol. In this case the whole of the extracted phosphatide, since it is alcohol-soluble, would be held to be "lecithin." Now these divergent results can be obtained from the same tissue, so that according to the treatment of the material one observer may find nothing but "lecithin" while another may find abundance of both "lecithin" and kephalin.

Again, it very often happens that "lecithin," which dissolves easily in alcohol immediately after its separation from the tissues, becomes partly alcohol-insoluble even when preserved in a carefully evacuated brown glass desiccator. The alcohol-insoluble substance formed has the properties and general composition of kephalin. This change in solubility is much more in evidence if the organic solvents used are not quite pure, and in general the greater care taken to purify the various organic reagents—ether, acetone, etc.—the more "lecithin" is obtained. Particularly does this appear to be the case with ether.

In one experiment bearing on this point heart tissue was extracted with alcohol in the ordinary way: the residue was taken up in ordinary "good" ether as supplied by the dealers. Another portion was treated in exactly the same way, ether from the same source being used, but only after careful purification. Using ordinary ether a considerable amount of the phosphatide

ultimately obtained was insoluble in alcohol and part of the "lecithin" fraction became alcohol-insoluble after a week; on the other hand the specimen in which purified ether was used gave nearly all "lecithin" which, up to the present time (six weeks after preparation), remains quite soluble in alcohol.

From these anomalous results it is easy to explain the divergence of opinion with regard to the occurrence of kephalin in the tissues.

Thus, Erlandsen in heart muscle could obtain no trace of kephalin [1907], while Koch and Woods [1905] found kephalin in the heart tissue in excess of the lecithin in the proportion of 100 to 61. In many other cases these observers found more kephalin than lecithin as the following figures show.

Substance analysed	Total phosphatide per cent.	Lecithin per cent.	Kephalin per cent.
Egg yolk	9·9	3	6·9
Pancreas	1·89	0·59	1·3
Milk (cow)	0·086	0·049	0·037
Liver	2·3	0·8	1·5
Kidney	2·6	1·2	1·4

How are we to explain these results? The explanation appears to be that kephalin, when rapidly extracted from fresh tissues, is soluble in an alcoholic solution of "lecithin." Its insolubility is probably brought about by oxidation, so that when impure solvents are used or precautions to prevent chemical changes are not taken, some of the kephalin becomes insoluble. If these oxidation changes are very marked some of the lecithin may also become insoluble so that the "kephalin" represents in this case a mixture of true lecithin and kephalin; it would appear however that true lecithin is much less liable than kephalin to become alcohol-insoluble.

A good deal of controversy exists with regard to the question whether kephalin is absolutely or relatively insoluble in alcohol. It is obvious, however, that kephalin is not entirely alcohol-insoluble, more especially in the presence of other phosphatides. If, therefore, a tissue is treated with alcohol, two monamino-monophosphatides are extracted—lecithin and kephalin—but it depends on circumstances whether the kephalin is insoluble in the alcohol or remains dissolved in the alcoholic solution of lecithin. In the latter case the phosphatide would have all the general properties of "lecithin" and little or no kephalin would be obtained. The small amount of sphingomyelin present in the tissues would also be present in the alcohol extract.

From this it appears that the substance known as "lecithin" must in many, if not in all cases, be represented by a mixture of two substances—

kephalin which tends to become insoluble in alcohol, and a substance which remains alcohol-soluble—true lecithin. The lecithin of the text books is really a lecithin-kephalin mixture. While it is possible that the two substances may be present in some kind of feeble combination there is obviously no combination in the ordinary chemical sense; this is indicated by results to be described later. For the further discussion of the subject it is necessary to point out the chief differences which have been established between kephalin and true lecithin.

Kephalin. Kephalin owing to its insolubility in alcohol and the conditions just described can be obtained in the pure state. The pure substance seems to have the same general structure as true lecithin, but in the case of kephalin the base present is not choline but β-amino-ethyl alcohol. This base is difficult to isolate in that its combination with platinum chloride is soluble in alcohol, especially in the presence of glycerol or other products of phosphatide hydrolysis. The salt is precipitated from its alcoholic solution by ether. It may, however, be obtained as the platinum chloride or gold chloride double salt by careful concentration. It yields all its nitrogen when treated with nitrous acid and can be estimated quantitatively by Van Slyke's method. Its compounds are so soluble that a gravimetric method of estimation has not yet been found.

Kephalin is precipitated by cadmium chloride in alcohol solution, the *resulting combination being soluble in ether.*

True lecithin. Unlike kephalin, true lecithin has theoretically all its nitrogen in the form of choline. Hitherto it is probable that no one using the ordinary methods has succeeded in obtaining a lecithin in which the choline accounted for the whole of the nitrogen. That the "theoretical" compound does exist will be shown later. Like kephalin, lecithin is precipitated by cadmium chloride, but *under certain circumstances the precipitate is insoluble in ether.*

The composition of ordinary "lecithin."

The above observation, that ordinary lecithin is a mixture, is strengthened by the results of many investigators who found that the amount of choline obtainable from lecithin, and generally separated as the platinum compound, was not nearly sufficient to account for the whole of the nitrogen present. The figures given indicate sufficiently the variations found.

Investigator	Source of "lecithin"	Percentage of nitrogen of "lecithin" obtained as choline platinum chloride
Heffter [1891]	Liver	25
Erlandsen [1907]	Heart	42
Moruzzi [1908]	Egg yolk	77
MacLean	Heart	42 to 75
"	Egg yolk	65
"	Other specimen egg yolk	77
"	Trade lecithin (Riedel)	80
"	Trade lecithin (Kahlbaum)	90
Osborne and Wakeman [1915]	Milk	39·5

It is exceedingly suggestive that "lecithins" from the same source but prepared in different ways did not always contain the same percentage of choline, while control experiments with the same sample gave almost identical results. As a result of similar experiments the writer many years ago came to the conclusion that "the generally accepted lecithin formula in which the whole of the nitrogen was represented by a base—choline—is incorrect and can no longer be accepted" [1909, 1]. The correctness of this view was questioned by Malengreau and Prigent [1912] who, working with Kahlbaum's lecithin, obtained nearly the whole of the nitrogen as choline. They made use of the cadmium chloride salt of lecithin and hydrolysed for long periods by means of N/10 H_2SO_4. That certain trade lecithins do contain a large percentage of their nitrogen as choline had been already emphasised by me [1908], but in cases where I employed acid hydrolysis the acid used was much stronger than the above. I therefore thought it well to repeat the experiment of Malengreau and Prigent with "lecithins" prepared from heart and from eggs.

Method of separation of choline from lecithin.

The lecithin was boiled for several hours (14 to 72) with excess of N/10 H_2SO_4; the mixture was then allowed to cool, when the fatty acids separated out as a solid cake on the surface. This was separated by filtration. The fatty acids were then returned to the flask, some distilled water added and the whole boiled for several minutes. On cooling the acids which again separated as a solid cake were as before removed by filtration. This process was carried out from four to six times.

An aliquot part of the total liquor obtained was taken and neutralised with $Ba(OH)_2$ solution; the precipitate formed was separated by filtration and carefully washed many times with boiling water. The filtrate, acidified with hydrochloric acid, was evaporated to dryness on the water bath, the residue extracted with alcohol, the alcoholic solution filtered, made up

to a definite volume and divided into two equal parts. One part was used for estimation of the nitrogen, and the other for choline platinum chloride. The results given below were very similar to those obtained in my earlier experiments with stronger acids.

Source of lecithin	Time of hydrolysis in hours	Percentage of nitrogen of final filtrate obtained as choline platinum chloride
Kahlbaum's trade lecithin	14	91·4
„ „	35½	92
Heart (old specimen)	16	89
„ (by ether extraction)	32	63·8
„ (by subsequent alcohol extraction)	32	69
„ (direct alcohol extraction) ...	35½	72
„ (ether extraction)	35½	61·4
„ „ „ (control) ...	35½	62
„ (ether extraction, very fresh specimen)	30	52

The difference in choline content of "lecithins" from the same source is well brought out and is important in considering whether the "lecithin" is a mixture of true lecithin and kephalin or a combination. These results suggest that it is merely a mixture and not a combination, for in the latter case it is difficult to see how this variation in nitrogen content could arise.

Further evidence in support of this contention is furnished by an observation made several years ago [MacLean, 1909, 2] that when "lecithin" is precipitated by cadmium chloride, the cadmium compound obtained contains considerably more of its nitrogen as choline (in one case 10 % more) than the lecithin from which it was obtained. This observation has been confirmed by Eppler [1913] and also by Trier.

In my experiments, the cadmium chloride precipitate was washed with ether and it is possible that this, rather than the alcohol, was the cause of the difference. This, however, does not in any way influence the deduction to be drawn—that we are dealing with a mixture and not a compound.

There still remained the theoretical possibility that none of the methods used to estimate choline was satisfactory, and that all the nitrogen might still be present as choline.

These objections were answered by the discovery of Trier [1911], who found β-amino-ethyl alcohol $CH_2(NH_2).CH_2.OH$ among the products of hydrolysis of the phosphatides of bean meal (*Phaseolus vulgaris*). Later the same observer recovered amino-ethyl alcohol from egg lecithin, identifying it by means of the gold chloride combination [1912, 1, 1913]. Trier has shown that amino-ethyl alcohol when methylated with methyl iodide, methyl alcohol and potassium hydroxide is converted into choline [1912, 2].

Amino-ethyl alcohol was also discovered independently in egg lecithin by Eppler [1913] working in Thierfelder's Laboratory, and has been found several times by the writer in heart "lecithin" [MacLean, 1913, 2]. It has also been found by Trier in various other "lecithins" particularly of plant origin. The next point to determine was whether the whole of the nitrogen of "lecithin" can be accounted for by the choline and amino nitrogen present. The amino nitrogen may be assumed to be present as amino-ethyl alcohol, since there is abundant evidence that this base is always present in "lecithin"; this view is supported by the fact that no other base containing amino nitrogen can be obtained from lecithin. There are also many chemical considerations such as the extreme solubility of the platinum and gold chloride combinations of this substance which are suggestive. In hydrolysed lecithin, platinum chloride precipitates only choline while another combination with a base remains in solution in the filtrate. On carefully evaporating this filtrate, the only base obtained is the amino-ethyl alcohol salt which appears to be invariably present. Before giving details of the experiments performed to determine the distribution of nitrogen in "lecithin" the method by which the "lecithin" was prepared will be described, as this differs considerably from the ordinary methods and has been somewhat modified of late. The "lecithin" used in all the experiments to be described later, together with several "lecithins" already mentioned, was prepared in this way. The following are the chief steps, which may be somewhat varied according to circumstances.

Preparation of "Lecithin."

(1) The tissue used (heart, kidneys or egg yolk) was in the case of solid organs ground to a fine paste by a mincing machine, the paste spread out in a thin layer on a glass plate and dried at 30° by means of an air current generated by a fan. Sometimes the original paste was put into excess of alcohol for a few minutes, the alcohol separated by filtering through a cloth and the solid material finally pressed to a hard mass by a laboratory hand press. In either case, the dried tissue was passed through a coffee mill and a fine powder obtained.

(2) The dried tissue was thoroughly extracted several times (generally four to six) with excess of absolute alcohol, the various extracts mixed and concentrated to small bulk under reduced pressure at 40°.

(3) The residue was taken up with a small volume of ether, in which much of it remained insoluble. To the mixture, without any attempt at

filtration, acetone was added in excess and the precipitate obtained pounded together by a pestle and separated. The precipitate was again mixed with ether, precipitated by acetone and treated as before. By this process, which was repeated three times, all but traces of acetone-soluble bodies were removed.

(4) The precipitate from above was rubbed up in a mortar with a large excess of water and a good emulsion made; to the emulsion from one-quarter to one-third of its volume of acetone was added. On the addition of the acetone a large amount of substance separated in the form of large white flakes and floated on the surface of the liquid. This was removed partly by means of a glass spatula, and partly by filtration. It was again emulsified and precipitated three times more. The filtrate obtained from the first emulsification always contained much soluble matter—the nitrogenous impurity previously referred to—but after the first treatment only traces of this impurity were found in the liquid.

The solid substance which separated was now dried by treating it several times with fresh additions of acetone. Finally, as much acetone as possible was pressed out of the mass by means of a pestle and the whole taken up in ether, in which it was still partly insoluble, forming an opalescent mixture.

(5) The ether mixture was centrifuged when a clear supernatant fluid and a white precipitate were obtained. This white precipitate was found to be impure sphingomyelin. The ethereal solution was decanted off and treated with excess of acetone. The resulting precipitate was again taken up with ether when an almost clear solution was obtained. Centrifuging was repeated as before and the clear supernatant ethereal solution again precipitated with acetone. Generally it was only necessary to centrifuge twice, but if the phosphatide was not quite soluble in ether after a second treatment the process was repeated until a precipitate was obtained which was quite soluble in ether.

(6) The substance was now dissolved in alcohol, and if the solution was not quite clear it was allowed to stand for some time until the insoluble substance (crude kephalin) settled on the bottom of the flask. The amount of alcohol-soluble substance obtained depended on circumstances already explained. After separation of the alcohol-insoluble part by decantation the solution was filtered.

(7) The solution was now evaporated under reduced pressure at 40°, the residue taken up with ether, and the phosphatide separated from the

ethereal solution by acetone. The precipitate obtained gave a perfectly clear solution with ether and with alcohol. It was treated with acetone several times and dried in the desiccator *in vacuo* over sulphuric acid. This substance had all the properties of "lecithin." The samples obtained contained about 1·85 % of nitrogen and 4 % of phosphorus and had a N : P ratio of approximately 1 : 1.

Distribution of nitrogen in "lecithin."

At first, attempts were made to estimate the amount of nitrogen obtained by Van Slyke's method [1912], using the unhydrolysed lecithin. These experiments were rendered difficult, partly on account of the frothing which resulted, and partly owing to the difficulty of finding a suitable solvent for the lecithin. The solvent used was strong acetic acid, but it was found that after the preliminary shaking of the sodium nitrite and acetic acid according to Van Slyke's instructions, the addition of acetic acid (instead of the usual aqueous solvent or very weak acid or alkali) acted in such a way as to give a good deal of gas which was not taken up by the permanganate absorbing mixture. This introduced an error which had to be allowed for by controls, but even then the results were not satisfactory. In spite of these disadvantages, however, the numbers obtained are very suggestive. On hydrolysing the lecithin and then using the liquid containing the soluble products of hydrolysis no difficulty whatever was experienced.

The choline was estimated by hydrolysis with weak acid followed by the steps already described.

Experiments with whole "lecithin."

(1) *Amino nitrogen.* After dissolving the "lecithin" in strong acetic acid the solution was allowed to stand for 24 hours; unless this was done concordant results could not be obtained, for a fresh solution of "lecithin" in acetic acid invariably gave too high results. The estimation was done exactly as described by Van Slyke, secondary octyl alcohol being used to prevent frothing as much as possible.

Experiment. 2·35 g. "lecithin" dissolved in 25 cc. strong acetic acid; different amounts of this were taken and amino nitrogen estimated.

(1) 5 cc. (equal to 0·47 g. "lecithin") = 4·9 cc. nitrogen at 20°, 769.
(2) 5 cc. (equal to 0·47 g. "lecithin") = 4·95 cc. nitrogen at 20°, 769.
(3) 4 cc. (equal to 0·376 g. "lecithin") = 3·9 cc. nitrogen at 20°, 769.

Therefore 1 g. "lecithin" contains 6·0097 mg. N as amino nitrogen = 31·4 %.

(2) *Choline nitrogen.* 0·9714 g. "lecithin" boiled for several hours with N/10 H_2SO_4, filtered, treated with Ba $(OH)_2$, filtered, residue taken up with alcohol and made up to 25 cc.

10 cc. gave 0·1040 g. choline Pt. chloride = 66 % of total nitrogen.

The nitrogen in this sample was distributed as follows.

N in fatty acids	=	4 %
N as choline	=	66 %
N in amino form	=	31·4 %
Total	=	101·4 %

Other results obtained in this way are shown in the table.

No.	Amino nitrogen found in percentage of total nitrogen	Choline nitrogen found in percentage of total nitrogen	Nitrogen retained by fatty acids in percentage of total nitrogen	Percentage of total nitrogen accounted for
1	30	68·6	3	101·6
2	38·2	63	4·4	105·6
3	28	64	6	98
4	30	68	6	104

Although these results are not altogether satisfactory, it must be remembered that there are great difficulties in getting exact quantitative results when using platinum chloride as a precipitant for choline; also the very small amount of nitrogen retained in the fatty acids is naturally difficult to estimate accurately. When to these are added the difficulties experienced in dealing with whole "lecithin" in Van Slyke's apparatus the results are as accurate as could be reasonably expected. They suggest that all the nitrogen of "lecithin" is present, partly as choline, and partly in the amino form—as amino-ethyl alcohol. This is better brought out in the next set of experiments, where hydrolysed lecithin was used.

Experiments with hydrolysed "lecithin."

In these experiments hydrolysis was carried out with dilute hydrochloric or sulphuric acid. Since the fatty acids always retain a small amount of nitrogen, and the nature of this small fraction of the total is difficult to ascertain, the investigation was limited to the determination and distribution of the filtrate nitrogen. On account of technical considerations it was found most convenient in some cases to estimate the choline in one portion of the lecithin, a separate portion of the same sample being used for the amino-ethyl alcohol determination. This was done in two experiments. In other cases aliquot parts of the same filtrate were taken.

(1) *Amino nitrogen.* For the determination of this fraction hydrochloric acid was used. About 1 g. of the "lecithin" was boiled with excess of the acid for several hours. After separation of the fatty acids they were thoroughly washed several times with hot water, separated by filtration, and the combined extracts evaporated to a small bulk on the water bath. The hydrochloric acid was then nearly neutralised with sodium hydroxide, the liquid filtered, the residue extracted with water and the total filtrates made up to a definite volume (10–20 cc.). Of this 2 cc. were taken and the amino nitrogen estimated by Van Slyke's micro-apparatus.

(2) *Choline nitrogen.* For the quantitative determination of choline N/10 H_2SO_4 was used, the process being carried out in the manner already described. The results of two experiments are given below.

No.	Source of Lecithin	Percentage of filtrate nitrogen recovered as choline	Percentage of filtrate nitrogen recovered as amino-ethyl alcohol	Percentage of nitrogen accounted for
1	Egg yolk	70	31	101
2	"	66·4	35·8	102·2

Experiments with "lecithin," using aliquot parts of the same filtrate.

1·0935 g. lecithin were boiled with 100 cc. N/5 H_2SO_4 for 48 hours. After separation of the fatty acids the usual manipulations were performed and a final alcoholic filtrate of 25 cc. obtained. Separate portions of this were taken for the determination of (1) choline, (2) amino-ethyl alcohol, (3) total nitrogen.

(1) *Determination of choline.* To 10 cc. of filtrate an alcoholic solution of platinum chloride was added. The precipitate obtained after drying to constant weight at 105° weighed 0·115 g. This figure was obtained after deducting the weight of the double salt of amino-ethyl alcohol platinum chloride which was present to the extent represented by 0·07 mg. amino-nitrogen.

(2) *Determination of amino-nitrogen by Van Slyke's method.* 10 cc. alcoholic filtrate were acidified with hydrochloric acid and carefully evaporated to dryness. The residue was dissolved in 2·5 cc. distilled water, 2 cc. of this being used for the determination.

2 cc. aqueous solution gave 2·55 cc. N at 20° and 755,
i.e. 10 cc. contained 1·802 mg. amino-nitrogen.

(3) *Determination of total nitrogen.* 3·5 cc. alcoholic filtrate required 1·75 cc. N/10 H_2SO_4 = 2·45 mg. N, i.e. 10 cc. = 7 mg. N.

From these figures:

Total nitrogen in filtrate = 17·5 mg.

Nitrogen present as choline = 13·07 mg.
Nitrogen present as amino-ethyl alcohol = 4·505 mg.
Total 17·575 mg.

In this case the nitrogen of the filtrate was all accounted for, 74·6 % being present as choline and 25·7 % as amino-ethyl alcohol.

Somewhat similar results were obtained in other experiments as shown by the table.

No.	Source of Lecithin	Total nitrogen of filtrate in mg.	Pt. chloride obtained in g. (calculated from amount obtained from aliquot part)	Mg. nitrogen present in choline chloride obtained	Mg. nitrogen present as amino-ethyl alcohol	Percentage nitrogen as choline	Percentage nitrogen as amino-ethyl alcohol	Percentage of total filtrate nitrogen accounted for
1	Egg yolk	16·5	0·2860	13	3·9	78·8	23·6	102·4
2	Ox heart	20·2	0·3298	15	4·95	74·2	24·5	98·7
3	Horse kidney	23	0·3364	15·3	8	66·5	34·8	101·3

From these results there can be no doubt that the whole of the water-soluble nitrogen of lecithin is present in the form of choline and amino-ethyl alcohol. It is probable that the nitrogen retained in the fatty acids is also in the amino form though some of this may be due to traces of impurity in the lecithin; it is worth noting that in many cases the nitrogen content of the lecithin is often slightly higher than is required to give a N : P ratio of 1 : 1. At any rate the part retained by the fatty acids is small.

When an alcoholic solution of choline containing amino-ethyl alcohol is precipitated by platinum chloride it frequently happens, if the solutions are concentrated, that some amino-ethyl alcohol platinum chloride is carried down with the choline double salt. This was found to be the case to a very slight extent in the above experiments. Here the amino nitrogen actually present in the platinum chloride precipitate was estimated and due allowance made. In this connexion, it is best not to have the alcoholic choline solution too concentrated; again the precipitate should be very thoroughly washed with absolute alcohol before drying. It must be dried at 105° to remove water of crystallisation. In pure alcoholic solution amino-ethyl alcohol gives a distinct precipitate with platinum chloride, especially if the latter is added in excess, but in the presence of glycerol and other impurities the double salt tends to remain in solution[1].

[1] In this connexion Osborne and Wakeman [1915] record the fact that the final alcoholic extract of some hydrolysed lecithin yielded on the addition of platinum chloride only a certain

Experiments with phosphatides differing in solubility in alcohol.

When lecithin is prepared from a tissue and kept for some time, a good deal of it tends gradually to become insoluble in alcohol while some remains easily soluble as already described. Between these two extremes, however, fractions of varying solubility in alcohol can be obtained.

Thus, in one experiment about 20 g. of some old heart lecithin were rubbed up three times in a mortar with about 80 cc. alcohol. The alcohol was separated by filtration and contained lecithin readily soluble in alcohol which was separated and dried in the usual way: Fraction I. The solid residue from above was now extracted thoroughly with excess of alcohol, and a lecithin fraction rather less soluble in alcohol obtained: Fraction II.

The alcohol-insoluble part remaining was dissolved in ether and great excess of alcohol added. A great deal was precipitated but some remained in solution: Fraction III.

The precipitate was dissolved in ether and precipitated by acetone. This was practically alcohol-insoluble kephalin: Fraction IV.

From the results already obtained it was to be expected that the amount of choline present should decrease and the amount of amino-ethyl alcohol increase in proportion to the relative insolubility in alcohol of the different fractions.

This proved to be the case; the results given were obtained by the methods already described.

Phosphatide	Percentage of nitrogen as choline	Percentage of nitrogen as amino-ethyl alcohol
Fraction I	67·7	33
„ II	—	38·2
„ III	43	55·3
„ IV	—	81

These figures substantiate the statement previously made that "lecithin" is a variable *mixture* and not a *compound*. Osborne and Wakeman in the communication already referred to state that the ether-soluble milk phosphatide obtained by them may be a mixture or combination of two substances, one yielding choline and the other a base containing amino-nitrogen. They intend to fractionate the substance to settle this question. In the light of the above observations it is probable that this phosphatide will be found

fraction of the total choline; after evaporating the filtrate, taking the residue up in alcohol and adding more platinum chloride another precipitate was obtained. This was repeated four times. It is probable, however, that some of these latter precipitates at any rate contained amino-ethyl alcohol platinum chloride.

to fall into line with other phosphatides in that it consists of a mixture of true lecithin and kephalin.

Attempts were now made to separate "lecithin" into its component parts. After many unsuccessful experiments I succeeded in doing this, and obtained a true lecithin with all its nitrogen present as choline.

Separation of "lecithin" into its components.

The lecithin used was derived from ox hearts and from eggs, the extraction and purification being carried through as quickly as possible.

The first experiments described were conducted with ox heart lecithin which was at first completely soluble in alcohol. On analysis for nitrogen, phosphorus and distribution of nitrogen, it gave the following results.

Nitrogen (Kjeldahl). 0·8007 g. required 10·85 cc. N/10 H_2SO_4 = 1·89 % N.

Phosphorus (Neumann). 0·5990 g. required 43·7 cc. N/2 NaOH = 4·04 % P.

N : P = 1·03 : 1.

Distribution of nitrogen.

1·3202 g. lecithin boiled with 120 cc. N/10 H_2SO_4 for 48 hours gave, after the usual treatment and addition of platinum chloride to the final alcoholic solution, 0·3673 g. choline platinum chloride (platinum content = 31·63 %).

The fatty acids contained 0·448 mg. nitrogen.

From above figures:

Total nitrogen of 1·3202 g. "lecithin"	= 24·95 mg.
Nitrogen found in fatty acids	= 0·448 mg.
Nitrogen of filtrate (by difference)	= 24·502 mg.

Now 24·502 mg. nitrogen = 0·5387 mg. choline platinum chloride and choline platinum chloride obtained = 0·3673 mg., i.e. 68 % of filtrate nitrogen is derived from choline.

By Van Slyke's method the filtrate from choline platinum chloride was found to contain 28 % of its nitrogen in amino form.

After standing for four days in an evacuated desiccator this "lecithin" formed a slightly opalescent alcoholic solution which on standing for some time deposited a very small amount of a sticky brown substance. The

solution was filtered and treated with excess of an alcoholic solution of cadmium chloride.

The resulting precipitate of cadmium chloride compound was thoroughly washed with alcohol, the latter being finally drained off by means of the suction pump. The precipitate was then dried *in vacuo*.

The dried powder was now ground up in a mortar with excess of pure dry ether. By this means it was hoped to extract the kephalin fraction, since kephalin cadmium chloride is said to be soluble in ether, while, on the other hand, the corresponding lecithin salt is supposed to be insoluble.

Here, however, the whole of the cadmium chloride precipitate dissolved in ether to form an almost clear solution. The ether was partly evaporated under reduced pressure; the concentrated solution obtained being spread out in a thin layer under a fan and freed from ether. The residue was taken up with fresh ordinary ether which had not been specially purified; a good deal remained in suspension as a brownish white powder. This was separated by the centrifuge and dried; it was then thoroughly ground in a mortar, extracted with ether several times and finally obtained as a white powder.

The ether-soluble part was freed from ether and the residue finely powdered. On treating a fraction of this with ether it was found to be entirely ether-soluble.

The cadmium chloride compound was now divided into two parts:

A. An ether-insoluble part forming a white powder.

B. An ether-soluble part forming a brownish white powder.

Treatment of ether-insoluble fraction A.

This fraction was further purified by twice recrystallising it from a mixture consisting of ethyl acetate, 2 parts, 80 % alcohol, 1 part.

The compound was dissolved by heat in this mixture and on cooling the greater part separated as a white precipitate, which under the microscope showed beautiful white clusters of well-formed needles arranged in stars. These crystals were washed with ethyl acetate and with ether and dried.

Analysis of crystals.

Nitrogen (Kjeldahl). 0·2998 g. required 3·1 cc. N/10 H_2SO_4 = 1·45 % N.

Phosphorus (Neumann). 0·1310 g. required 7·45 cc. N/2 NaOH = 3·15 % P.

N : P = 1·02 : 1.

Distribution of nitrogen in crystals.

0·5896 g. was boiled with excess of N/10 H_2SO_4 for 42 hours, the final alcoholic solution containing the soluble nitrogen being obtained in the usual way. Filtrate gave 0·1644 g. platinum chloride salt (Pt. content 31·55 %). Fatty acids contained 0·95 mg. nitrogen.

From above figures:

Total nitrogen of 0·5896 g. compound	= 8·54 mg.
Nitrogen found in fatty acids	= 0·95 mg.
Filtrate nitrogen (by difference)	= 7·59 mg.

Now 7·59 mg. nitrogen = 0·1669 g. choline platinum chloride and choline platinum chloride obtained = 0·1644 g. i.e. 98·5 % of filtrate N is derived from choline.

The filtrate from the platinum chloride precipitate gave no amino nitrogen by Van Slyke's method. The whole of the filtrate nitrogen was therefore removed by platinum chloride.

With the exception of the variable amount of nitrogen always retained by the fatty acids this powder contained all its nitrogen as choline, while the "lecithin" from which it was derived contained only 68 % of its soluble nitrogen as choline.

This separation proves that "lecithin" is a mixture from which its choline-lecithin component can be separated by making use of the insolubility of its cadmium chloride salt in ether.

Investigation of the ether-soluble part of the cadmium chloride precipitate (Fraction *B* above) showed that it still contained some choline. Since the whole of the cadmium chloride precipitate originally obtained from lecithin dissolved in ether on the first attempt at separation, this result was to be expected. Choline to the extent of about 30 % of the total nitrogen was found. Attempts to reduce the amount of choline were frustrated by the extreme solubility of the salt in ether, and it is probable that although true lecithin cadmium chloride is insoluble in ether, it dissolves to a certain extent in an ethereal solution of kephalin cadmium chloride.

A fresh sample of lecithin was now obtained from hearts, and the cadmium chloride compound prepared as before. Here the fractionation of the salt gave rise to great difficulties, for when treated with ether an opalescent solution was formed which it was impossible to filter or centrifuge. Evaporating the ether and again taking up the dried substance with ether

—a procedure which was very effective in the former separation described—proved quite useless here. It was found, however, that if the dried material was made into a thick paste with alcohol, and ether containing 2 % alcohol was used, an opalescent solution was obtained which filtered very well. By this means the compound was divided into two parts as before.

A. Fraction insoluble in ether = true lecithin.

B. Fraction soluble in ether = kephalin contaminated with lecithin.

Now Thudichum [1884] makes the statement that lecithin cadmium chloride is soluble in cold benzene, and that the part which remains insoluble in the cold but dissolves on heating, to fall out again on cooling, is not lecithin but a substance which he calls paramyelin. An attempt at fractionating the white powder *A* by means of benzene was therefore made. This, however, was unsatisfactory and in my case the process did not appear to be of much value. When cold benzene was added to the powder very little indeed appeared to dissolve, but on heating the mixture nearly all the solid disappeared. On leaving this solution to stand in the ice chest for some hours an opalescent layer formed at the bottom with a clear liquid above. On filtration an absolutely clear benzene solution was obtained, while a fair amount of a semitransparent gelatinous substance was left on the filter. When this was treated with hot benzene part of it dissolved but did not form a clear solution. On again leaving it to stand at low temperature a marked deposit formed. This deposit constituted fully one half of the total substance.

When treated with hot benzene some of this apparently goes into solution, and the process of separation of lecithin cadmium chloride into benzene-soluble and benzene-insoluble fractions appears to be unsatisfactory. It seems that practically the whole can be got into solution if sufficiently large volumes of benzene are used and any differences of solubility that may exist are probably due to slight oxidation or other changes; the "insoluble" and "soluble" fractions are probably the same substance.

The ether-insoluble fraction was now divided into

(1) Fraction remaining soluble in cold benzene (original solution being accomplished by means of heat),

(2) Fraction insoluble in cold benzene (soluble more or less in hot benzene).

The cold benzene solution was treated with twice its volume of alcohol and the precipitate obtained separated by the centrifuge.

The cold benzene-insoluble fraction (2) was spread out in a thin film under a fan and dried at room temperature. Both substances were then dissolved in the ethyl acetate-alcohol mixture as before and white crystalline precipitates obtained. The following scheme represents the fractionation of the original "lecithin."

Lecithin → $CdCl_2$ compound { Ether-insoluble fraction { Benzene-soluble fraction (lecithin); Benzene-insoluble fraction (lecithin) }; Ether-soluble fraction (impure kephalin) }

The distribution of the nitrogen in these compounds is given in the table. Hydrolysis[1] was carried out in the usual way and aliquot parts of the filtrate used for choline and for total nitrogen. The amino nitrogen was estimated in the filtrate after the separation of choline as the platinum salt. No account is taken of the nitrogen retained by the fatty acids or other precipitate.

The numbers refer to the total nitrogen of the filtrate.

No.	Substance	Percentage nitrogen as choline	Percentage nitrogen in amino form	Percentage of total filtrate nitrogen accounted for
1.	Lecithin	62	37·2	99·2
2.	Cadmium chloride lecithin, before ether	65	33	98
3.	Ether-insoluble fraction (benzene-soluble)	94	3	97
4.	Ether-insoluble fraction (benzene-insoluble) ...	99	None	99
5.	Ether-soluble fraction	45	54	99

Here the two fractions (3 and 4) contained all their nitrogen (with the exception of a trace in No. 3) as choline. A cadmium chloride compound of a true choline-lecithin has therefore been obtained from an original lecithin which contained only 62 % of its nitrogen as choline.

It is impossible to get the kephalin part quite free from true lecithin by this process, but kephalin can be obtained in the pure state by treatment with alcohol and suitable purification, and as such has been isolated and

[1] Considerable difficulty was sometimes experienced in hydrolysing cadmium chloride compounds of phosphatides with N/10 H_2SO_4. Often a great deal of the nitrogen was retained in the precipitates obtained during the preparation of the final alcoholic extract for precipitation by platinum chloride. Sometimes the whole of the nitrogen (with the exception of the small amount retained in the fatty acids) was found in the filtrate. The reason of these anomalous results I have been unable to ascertain. When lecithin itself was used no difficulty of this kind was encountered.

analysed by many investigators. It contains the whole of its nitrogen as amino-ethyl alcohol.

So far, only the cadmium chloride salt of true lecithin had been isolated, but on separation of the free phosphatide by means of ammonium carbonate, free lecithin was obtained which had all its nitrogen in the form of choline. This particular sample of lecithin was derived from egg yolk and as its method of preparation differed considerably in certain respects from the methods already given, this will now be described.

Preparation of a sample of lecithin containing all its nitrogen as choline.

Dried egg yolk was powdered and extracted with alcohol: to the alcoholic extract was added excess of alcoholic solution of cadmium chloride.

The resulting precipitate was washed with alcohol and then rubbed up with about fifteen times its volume of ether containing a trace of alcohol. A dense opalescent mixture was obtained which on centrifuging separated into a brownish deposit and clear supernatant liquid. The deposit was thoroughly washed with ether, dried and decomposed by boiling in alcohol with ammonium carbonate according to the method recommended by Bergell [1900].

The alcoholic solution was concentrated, the residue taken up with ether and excess of acetone added. The precipitate obtained was emulsified with water and treated by the usual process for the purification of lecithin [MacLean, 1912]. The lecithin was dissolved in alcohol and again precipitated by cadmium chloride. The double compound obtained was recrystallised from the ethyl acetate mixture already described. Beautiful white feathery crystals of pure lecithin cadmium chloride were thus obtained.

Analysis of pure lecithin cadmium chloride (egg).

Nitrogen (Kjeldahl). 0·5604 g. required 5·85 cc. N/10 H_2SO_4 = 1·46 % N.

Phosphorus (Neumann). 0·2600 g. required 15·8 cc. N/2 NaOH = 3·37 % P.

N : P = 1 : 1·04.

Test for amino nitrogen.

1·8615 g. were boiled for 10 hours with dilute hydrochloric acid (96 cc. H_2O + 4 cc. conctd. HCl); after cooling the fatty acids were separated by filtration, the filtrate evaporated to a small bulk on the water bath and nearly

neutralised with alkali. The whole was then made up to 20 cc. Of this 2 cc. were used for each experiment.

The following results were obtained by Van Slyke's method.

Controls with reagents { (1) 0·12 cc. nitrogen; (2) 0·13 cc. „ } at 752 and 20°.

Experiments { (1) 0·14 cc. nitrogen; (2) 0·15 cc. „; (3) 0·15 cc. „ } at 752 and 20°.

Average 0·146 cc. nitrogen.

2 cc. therefore contained 0·021 cc. nitrogen and from this 1·8615 g. (20 cc. solution) gives 0·210 cc. nitrogen = 0·117 mg. nitrogen in amino form.

Total nitrogen in 1·8615 g. compound = 27·17 mg.
Total nitrogen in filtrate = 26·80 mg.
Retained by fatty acids, etc. = 0·37 mg.

From above figures:

Percentage of total nitrogen in amino form = 0·430
Percentage of filtrate nitrogen in amino form = 0·437

Separation of pure lecithin from its cadmium chloride compound.

This was done as already described by ammonium carbonate. The substance obtained was thoroughly purified by means of acetone and water and finally dried *in vacuo* over H_2SO_4.

Analysis.

Nitrogen (Kjeldahl). 0·3747 g. required 5 cc. N/10 H_2SO_4 = 1·87 % N.
Phosphorus (Neumann). 0·2710 g. required 20·3 cc. N/2 NaOH = 4·15 % P.

N : P = 1 : 1.

Estimation of choline.

1·8426 g. were boiled with 150 cc. N/10 H_2SO_4 and after the necessary manipulations 25 cc. of a final alcoholic solution obtained.

10 cc. of this solution gave 0·2993 g. platinum salt (platinum content 31·7), therefore total filtrate = 0·7483 g. platinum salt.

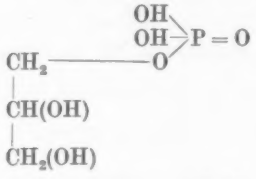
Now total nitrogen of 1·8426 g. substance = 34·46 mg. = 0·7581 g. choline platinum chloride; obtained 0·7483 g. choline platinum chloride, i.e. 98·7 % of total nitrogen is derived from choline.

Since, however, a small amount of nitrogen is always retained in the fatty acids the whole of the soluble nitrogen of this specimen may be regarded as choline. The platinum chloride removed every trace of the filtrate nitrogen.

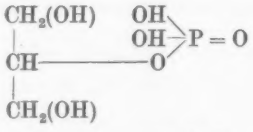
Some considerations on the chemical constitution of "lecithin."

Fatty acids. The fact that lecithin has been shown to consist of two components throws some light on the difficulties experienced by many investigators when endeavouring to ascertain the nature of the fatty acids present. Theoretically only two acid radicles exist in the molecule, and separation and identification ought to be easy, but, generally, mixtures are obtained which appear to consist of more than two acids. It is hoped, however, that examination of the true lecithin obtained by the processes described will give better results.

Glycerophosphoric acid. From the chemical standpoint this acid can exist in two modifications—the α- and β-forms.



α-form (unsymmetrical)



β-form (symmetrical)

The α-form contains an asymmetric carbon atom and is therefore optically active, while the β-variety is inactive.

After the discovery by Willstätter and Lüdecke [1904] that the glycerophosphoric acid of egg lecithin was optically active, rotating the plane of polarisation of polarised light to the left, it was generally assumed that the acid present in lecithin was α-glycerophosphoric acid, though Tutin and Hann [1906] disputed this and held that lecithin glycerophosphoric acid consisted of a mixture of the α- and β-forms. Recently Grimbert and Bailly [1915] claim to have definitely shown that egg lecithin is a mixture of at least two isomers containing the symmetrical and unsymmetrical varieties of glycerophosphoric acid.

It is possible that the true lecithin part of "lecithin," containing all its nitrogen as choline, may have one form of this acid, while the kephalin part may have the other form. Some experiments bearing on this point were begun some time ago but have had to be stopped for the present. I hope, however, to take up this point in the future and to settle some of these problems in a later communication.

SUMMARY.

(1) When phosphatides are extracted from tissues by means of alcohol they invariably contain large amounts of a nitrogenous impurity. This is very difficult to remove by any of the ordinary methods for preparing "lecithin" and many bodies described as new substances are simply "lecithin" contaminated with this body.

(2) This nitrogenous impurity is very complex chemically and contains bodies of a purine nature.

(3) The whole of the nitrogen of "lecithin" is accounted for by the choline and amino nitrogen present.

(4) "Lecithin" is a mixture in variable proportions of two somewhat similar substances, one having the whole of its nitrogen in the form of choline —true lecithin—while the other contains no choline but has all its nitrogen represented by amino-ethyl alcohol—kephalin.

(5) Reasons for the divergent statements as to the occurrence of kephalin in different tissues are given.

(6) By fractionation of the cadmium chloride salt of "lecithin" with ether, "lecithin" has been divided into its two components—true lecithin and kephalin. True lecithin in which every trace of the nitrogen was present as choline was obtained. The kephalin fraction contained a certain amount of choline, but had the greater part of its nitrogen as amino-ethyl alcohol.

(7) Various points on the mode of occurrence, distribution and purification of the phosphatides are discussed.

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XXXVII. THE REDUCING ENZYME OF *BACILLUS COLI COMMUNIS.*

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A reducing enzyme is fairly widespread in the tissues of animals and plants and has of late received the attention of several workers in the province of enzymic chemistry. The results obtained independently by some of the investigators seem to show that the so-called reductase is not a single principle but a system. Thus Bach [1911] working on the reducing enzyme in the calf's liver was able by treating the tissue with a 2 % solution of sodium fluoride and pressing it through a cloth to obtain an inactive filtrate and a residue which when emulsified with water either did not reduce methylene blue at all or did it extremely slowly. On the addition of 1 % of acetaldehyde to the emulsified residue the reducing activity was restored. Harden and Norris too [1914] showed that dried yeast and zymin lost their power of reducing methylene blue and sodium selenite when washed. This loss of the reducing power they found could be restored by the addition of the washings, bouillon and some aldehydes. Recently [1915] they have observed a similar phenomenon with the reducing enzyme of muscle. Schardinger's observation that the addition of acetaldehyde and formaldehyde to milk imparts to it the power of reducing methylene blue to its leuco-base is another illustration of the complexity of reductase.

The authors find that *B. coli communis* (Escherich) when washed will not reduce methylene blue, but that it acquires the power of reduction on the addition to it of various reagents incapable by themselves of effecting the reduction. This property of the organism in question was investigated and the results obtained form the subject of this communication.

The following technique was adopted in the preparation of the bacterial emulsion used in the experiments. Roux bottles containing nutrient agar were inoculated with a 24 hours' broth culture of *B. coli*. The organism was then grown for 48 hours at 37° after which period it was washed off the agar with saline, a glass scraper being used in order to ensure a more thorough removal of the growth. The resulting emulsion was then centrifuged and after removing the supernatant saline fresh salt solution was added, the organisms stirred up, and again centrifuged. This process of washing was repeated two or three times and the residue was finally diluted with saline (the growth from six Roux bottles to 50 cc. of saline) and shaken up with glass beads in order to obtain an even suspension of the bacilli. The emulsion was then ready for use. With very few exceptions a fresh preparation was made daily. If kept in the cold room, however, the emulsion retained its reducing power for some days.

As already mentioned *B. coli* will not reduce methylene blue when washed in saline unless certain substances are added to it. A great number of reagents were tested for this property and a good many of these yielded positive results. The following list comprises the substances tried.

Positive.	*Negative.*
Washings	Acetaldehyde
Bouillon	Formalin
Sodium lactate	Lactose
Sodium pyruvate	Sorbitol
Sodium glycerate	Dulcitol
Glucose	Raffinose
Galactose	Erythrol
Laevulose	Rochelle salt
Mannose	Glycocoll
Maltose	Potassium oxalate
Arabinose	Anisaldehyde
Mannitol	Oenanthol
Sodium succinate	Phloroglucinol
Sodium formate	Propaldehyde
Alanine	Isovaleraldehyde
Asparagine	Cinnamyl aldehyde
Xylose	Salicylaldehyde
Inulin	Hydroxybutyric acid
Sodium acetate	Butyl alcohol
Isobutyric acid	Linolenic acid
Globulin	Sodium citrate
Albumin	Hydantoin
Horse serum	Creatine
Peptone (Witte)	Guanine hydrochloride
Liebig's Extract	Pyridine
	Hypoxanthine, Adenine, Xanthine (In suspension)

In carrying out the above experiments the following quantities were used: 1 cc. of the bacterial emulsion, 1 cc. of the reagent, 0·2 cc. methylene blue (5 cc. saturated solution diluted to 200 cc. with water). The mixtures were made up to 5 cc. with saline. The salts were employed in N/10 solution, while the other substances, with the exception of the washings, broth, and horse serum, were in 1 % solutions or suspension. All the reductions were carried out at 35–37°. The time taken to reduce the methylene blue varied with the reagent used. The results were regarded as negative when no reduction was observed within an hour. The addition of broth produced one of the highest rates of reduction; an addition of a very small quantity sufficed to impart the reducing power to the emulsion. This is shown by the following experiment:

Emulsion cc.	Broth cc.	Saline cc.	Methylene blue cc.	Time taken for reduction mins.
1	—	4	0·2	(no reduction)
1	0·1	3·9	0·2	20
1	0·2	3·8	0·2	14·5
1	0·3	3·7	0·2	12
1	0·4	3·6	0·2	11·5

It has been suggested that in the process of reduction water is split into H and O and in order that the reduction should occur, the presence of an acceptor for the oxygen is necessary. The great variety in the chemical character of the reagents in the list which gave positive results does not afford definite information as to the precise chemical properties requisite for an acceptor. Bach [1912] in connection with his work on the reductase in calf's liver, discussing the chemical nature of the acceptor or coferment, as he calls it, suggested the possible presence in all solutions capable of acting as acceptors of aldehydes formed from such degradation products of the metabolism of the proteins as α-amino acids. In support of his suggestion he refers to Strecker's observations that α-amino acids are oxidised at ordinary temperature by alloxan forming aldehydes of a series containing fewer carbon atoms and giving off at the same time CO_2 and NH_3, a process which might possibly occur in nature. The experiments quoted above however show that in the case of *B. coli* all the aldehydes tried with the exception of some of the sugars are incapable of acting as acceptors under the conditions employed. From the same experiments we can also conclude that the reducing enzyme in the organism in question differs to some extent from those present in the liver of the calf and in yeast, since in the former case according to Bach acetaldehyde can act as acceptor and

in the latter case according to Harden and Norris salicylaldehyde, benzaldehyde, anisaldehyde, and isovaleraldehyde are able to restore the reducing power to washed yeast.

The effect of the addition of increasing quantities of broth was next studied and it was found that the rate of reduction increased at first with the increase in the quantity of broth added until a certain optimum was reached, after which any further increase in the quantity of broth added decreased the rate of reduction. This is illustrated by the following experiment.

Emulsion cc.	Broth cc.	Saline cc.	Methylene blue cc.	Time of reduction mins.
1	—	4	0·2	(not reduced)
1	0·1	3·9	0·2	28·5
1	0·2	3·8	0·2	24
1	0·3	3·7	0·2	22·5
1	0·4	3·6	0·2	19
1	0·5	3·5	0·2	14·5
1	1	3	0·2	15
1	1·5	2·5	0·2	15·5
1	2	2	0·2	18
1	2·5	1·5	0·2	20
1	3	1	0·2	20·5
1	3·5	0·5	0·2	24

This decrease in the rate of reduction produced by increased addition of broth after a certain optimum is an observation of some interest. Palladin [1914] found that the addition of glycerol and pyridine to hefanol produced a deterrent effect on the activity of the reductase in it and that the extent of the retardation varied with the amount of the reagent added. He ascribes the fact to the displacement of the water by the glycerol and pyridine. Pyridine does not act as an acceptor when added to *B. coli*, glycerol on the other hand does, but its behaviour was observed to be similar to that of broth, since it has at first an activating influence which is proportional to the amount added, but after a certain point any increase in the amount of the reagent decreases the rate of reduction. This is shown in the following experiment.

Emulsion cc.	Glycerol 50% cc.	Saline cc.	Methylene blue cc.	Time of reduction mins.
1	—	4	0·2	(no reduction)
1	1	3	0·2	20
1	2	2	0·2	16·75
1	3	1	0·2	20·5
1	4	—	0·2	23

This experiment and also the one carried out with the broth suggest that both reagents possess an activating, as well as a retarding action on the reductase of *B. coli*. The experiments throw no direct light on the cause of the retarding influence, but at the same time they do not exclude Palladin's explanation. It is quite conceivable in this case that glycerol for instance can act chemically as an acceptor, while as it displaces the water owing to its physical condition it can also retard and even stop the action of the reducing system of which it forms a part (i.e. acceptor). It is also plain from the experimental data that initially the rate of reduction is influenced more by the activating than by the retarding factor, but as the quantities of the reagents added are increased the reverse takes place, and this accounts for the decrease in the rate after a certain amount of the reagent has been added.

It was of further interest to investigate how the time of reduction is influenced by varying the amounts of methylene blue and emulsion respectively, and accordingly experiments were instituted with that object. In the first set the amount of emulsion was kept constant and the quantity of methylene blue changed. In the second set the methylene blue was kept constant and the amount of emulsion changed. The following are the results obtained:

	Emulsion cc.	Broth cc.	Saline cc.	Methylene blue cc.	Time of reduction mins.
1.	1	1	2·9	0·1	19
	1	1	2·8	0·2	21·5
	1	1	2·7	0·3	25
	1	1	2·6	0·4	26·5
	1	1	2·5	0·5	29
2.	0·1	1	3·9	0·2	43
	0·3	1	3·7	0·2	20·5
	0·5	1	3·5	0·2	13
	1	1	3	0·2	7·5
	2	1	2	0·2	5

As it is seen from the figures the time taken to reduce increases as the quantity of methylene blue is increased, while in the case of the varying quantities of emulsion the time decreases with the increase in the amount of emulsion.

No essential difference in the results could be observed when some of the experiments were carried out anaerobically, i.e. by boiling the saline previous to using it and by displacing the atmosphere in the tubes and the dissolved oxygen in the emulsion by nitrogen.

SUMMARY.

1. *B. coli* does not reduce methylene blue when washed and suspended in saline, but does so on addition of certain reagents.

2. A wide variation in chemical nature is exhibited by the reagents capable of acting in this way.

3. The effect of the addition of various amounts of broth and glycerol respectively show that there are two factors concerned in the effect of those reagents which influence the process of reduction, i.e. an activating factor and a retarding one. The former is thought to be chemical while the second is possibly physical.

4. The time of reduction of a certain quantity of methylene blue decreases with increase in the amount of emulsion, while as the amount of methylene blue is increased for a constant amount of emulsion, the time of reduction increases.

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XXXVIII. ON THE DIAZO REACTIONS OF HISTIDINE AND TYROSINE.

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(*Received August 19th, 1915.*)

The test for histidine based on its property of forming a diazo compound, first described by H. Pauly [1904], is one of the most important reactions in the investigation of protein decomposition products. This diazo reaction can be carried out without any modification whether histidine is in the free state or bound up in a protein molecule. Its importance is, however, lessened considerably as it is not quite specific, since tyrosine, too, is capable of coupling with diazobenzenesulphonic acid in alkaline solution and yields a colouration so very similar to that given by histidine, that the differentiation of the two amino acids from each other by means of this reaction is a matter of extreme difficulty. On addition of diazobenzenesulphonic acid in sodium carbonate solution, histidine gives a dark cherry red colouration which, even on dilution with much water, retains its red tone and does not change into yellow: in acid solution it gives a pure orange colouration. As regards tyrosine, the colour in alkaline solution is like that of histidine, but of a less deep red, and, moreover, on dilution, a yellow colour appears, yielding finally an impure yellowish red colour.

Though Pauly was able to describe the difference of the diazo colours produced by histidine and tyrosine, as above, he thought it necessary to mention further, that it is sometimes not easy to distinguish the colours from each other.

Applying the diazo reaction as a test for methyltyrosine, E. Friedmann and S. Gutmann [1910] tried to distinguish the colouration given by this substance from that of histidine by taking into consideration not only the

colours of the solutions themselves, but also the colours with which these solutions dye filter paper. The histidine compound gives either a pure orange or a lemon yellow colour on paper, depending on the reaction of the solution being alkaline or acid respectively. The methyltyrosine compound stains paper red brown in alkaline, yellow brown in acid solution. Even using this method of differentiation for tyrosine, it is still scarcely possible to distinguish it from histidine by the diazo reaction.

Since, according to Pauly, among the protein cleavage products, histidine and tyrosine alone give the diazo reaction, it is quite evident that the reaction will be more useful in its application, if it be made specific for only one of these two amino acids.

Lately K. Inouye [1912] has succeeded with the following useful modification. He noticed that when tyrosine and histidine are submitted to the Schotten-Baumann reaction, the former is converted into its dibenzoyl derivative and loses its property of coupling with diazotised sulphanilic acid, while the latter, though forming its monobenzoyl compound, still maintains unaltered its ability of yielding a red colouration in the presence of the diazonium acid. He therefore treated the solution to be tested by means of the diazo reaction with benzoyl chloride and sodium carbonate, in order to prevent the tyrosine from combining with the diazo acid, and thus made the diazo reaction specific for histidine.

According to a recent paper by A. Kossel and S. Edlbacher [1915], when histidine loses its free carboxyl group, as, for instance, in its methyl ester, the imidazole ring to which the diazo reaction owes its appearance is readily opened on benzoylation with formation of a tribenzoyl-triamino-acid.

As regards histidine in a protein molecule, where it is to be found in a peptide linking united through its carboxyl group with other amino acids, it is most probable that by treatment with Schotten-Baumann's reagent an analogous transformation of histidine will take place, just as in the case of its methyl ester, resulting in the disappearance of the property of giving the diazo reaction.

In order to apply Inouye's modification of the histidine test to protein it is therefore necessary, before performing the test, to hydrolyse the protein by means of acid or trypsin, so as to obtain the histidine in the free state.

Recently, in applying some new methods of investigation to protein decomposition products, I met with some difficulty in distinguishing these two amino acids by means of the diazo reaction, since, owing to the special circumstances, neither Inouye's test for histidine, nor Millon's reaction could

be successfully used. At any rate it seemed to me of interest to discover a method of differentiating the diazo reaction as applied to tyrosine and histidine without recourse to benzoylation.

I find a ready distinction may be made by reducing the coloured solutions obtained by Pauly's reaction from these two amino acids. Although the two solutions obtained with diazobenzenesulphonic acid in sodium carbonate solution are at first almost identical in colour, yet on acidifying with hydrochloric acid and reducing with zinc dust, and then adding excess of ammonia, the resulting fluids are quite different in appearance.

The test is applied as follows: a few cubic centimetres of the solution to be tested are placed in a beaker and made alkaline with excess of sodium carbonate solution. A few centigrams of the freshly prepared diazobenzenesulphonic acid are dissolved in a few cubic centimetres of sodium carbonate solution. This solution must always be freshly prepared just before performing the test, as if it be allowed to stand longer than a quarter of an hour, the result of the reaction is sometimes dubious. The use of too large an excess of the reagent should be avoided, otherwise the solution remains slightly yellowish in colour after reduction and the test is occasionally rendered less distinct.

The reagent is now added to the amino acid solution in the beaker. Immediately, or not later than three minutes afterwards, the fluid assumes a dark red colouration if histidine or tyrosine be present. I will refer to this well-known diazo colouration as the primary colouration for the sake of distinguishing it from the colouration produced after reduction. As I have mentioned above, it is extremely difficult to judge by means of this primary colouration, whether the result is due to histidine or tyrosine.

To the coloured solution concentrated hydrochloric acid is added to make it distinctly acid, and this, in each case, changes the colour to a rather yellow shade of orange. The acid solution is reduced by zinc dust for a quarter of an hour or longer, a colourless solution always resulting. A few cubic centimetres of the reduced solution are now transferred to a test-tube by decantation, or, if necessary, by filtering, and then made strongly alkaline by addition of ammonia of about 25 % strength to the extent of approximately twice the bulk of the solution. Of course, the amount of ammonia necessary depends on the quantity of hydrochloric acid and of zinc used, but the above quantities will generally be found sufficient.

If the primary diazo reaction be due to histidine, the fluid yields immediately, or within a few minutes, a very characteristic golden yellow

colouration of great intensity which is permanent for a long time and is unaltered even on boiling. By the action of hydrogen peroxide on this golden yellow solution, the shade is turned into a beautiful lemon yellow.

The colouration produced by tyrosine in the above reaction is yellowish brown with a small quantity of ammonia, and on gradual addition of ammonia to the amount mentioned above is readily changed to a beautiful bright rose red colouration. This colour, too, is unaltered on boiling, but when left for a while becomes markedly yellow in tint and gradually changes to a reddish brown, which, however, may be reconverted into the former colour by further addition of ammonia or by shaking. Unlike that of histidine, the rose red colouration of tyrosine is remarkably unstable to the action of hydrogen peroxide, so that after oxidation by this substance, the solution will be colourless or, at most, of a faint impure rust colour.

The colouration obtained by the addition of ammonia after reduction of the primary coloured compounds will be called the secondary colouration. The intensity of the colour in the secondary reaction is always proportional to that of the primary colouration.

Converting thus the closely resembling primary colourations of the histidine and tyrosine diazonium compounds into the secondary, which differ so markedly in appearance—the one being golden yellow and the other rose red—one can now very readily distinguish the two amino acids by the diazo reaction followed by the reaction giving rise to the secondary colourations.

As regards histidine, the primary diazo reaction can be applied to a solution as dilute as one part of the substance to 100,000 parts of water; the secondary colouration, too, is obtained as a pale yellow even at this dilution. The peculiarly characteristic deep yellow, however, can be obtained only with a solution stronger than 1 : 20,000.

Glycerol, alanine, cystine, taurine, α-aminobutyric acid, valine, arginine, leucine, lysine, phenylalanine, tyrosine, tryptophane, histidine, proline, aspartic acid, glutamic acid and hippuric acid have been submitted to the test. Serine and oxyproline were not at my disposal.

Not one of these amino acids except histidine was able to produce a golden yellow colouration in the secondary reaction. It appears, therefore, that, at present, this secondary golden yellow colouration may be considered as being given by histidine alone among the protein decomposition products.

The secondary diazo reaction of tyrosine with production of a rose red colouration is so delicate that it can be carried out in a dilution of 1 : 10,000. With a dilution of 1 : 50,000 a faint rose red colouration is obtained. The

rose red colour, however, is not specific for tyrosine. Cystine, which was carefully purified and freed from tyrosine either by precipitating with phosphotungstic acid or by the method of R. H. A. Plimmer [1913] (treatment with alcohol and hydrochloric acid gas), gives the same colouration as tyrosine, though it is much less intense. Alanine and phenylalanine also slowly produce a faint pink colouration, but the solution must be rather strong. The same colour reaction is also given by asparagine with a delicacy of 1: 1000. All these colourations are destroyed by oxidation with hydrogen peroxide.

Some organic compounds other than protein cleavage products, for instance, hydroxy-acids such as lactic acid and malic acid, alcohols such as ethyl alcohol, glycol and glycerol, and also acetone, ether and oxamide, behave in a similar manner and yield the pink colouration, with or without the formation of a definite primary colour.

The rose red colouration due to tyrosine in the secondary reaction, therefore, is not specific for this amino acid, but taking into consideration the primary colouration in the diazo reaction which, in the intensity and rapidity of colour formation, is peculiar to tyrosine and histidine, one can be certain that the substance to be tested is really tyrosine when both the first and the second diazo colourations are produced in the particular manner described above. The diazo reaction of tyrosine has sometimes an advantage over Millon's reaction, for the former can be applied with ease, even where the latter is impossible, as for example in the presence of much chloride.

Making use now of the secondary diazo reaction it can be demonstrated clearly that tyrosine and histidine have different affinities for diazobenzenesulphonic acid.

Equimolecular quantities of these two amino acids were mixed together. To one portion of the solution an equimolecular proportion and to another twice this amount of pure diazobenzenesulphonic acid were added in sodium carbonate solution. When the secondary reaction was carried out, the former yielded a golden yellow, and the latter a yellowish brown colouration.

N/100 Histidine in cc.	2	2	2
N/100 Tyrosine in cc.	2	2	2
N/100 Diazobenzenesulphonic acid in cc.	2	4	6
Primary colouration	Brownish yellow	Brown yellow	Yellowish brown
Secondary colouration	Golden yellow	Yellowish brown	Yellowish brown
Colouration after oxidation with hydrogen peroxide	Lemon yellow	Lemon yellow	Lemon yellow

The different colourations produced in this experiment show distinctly that histidine has a greater affinity for diazobenzenesulphonic acid than

has tyrosine, so that it is the first to couple with the diazo acid. If more than sufficient diazo acid be present to combine with the histidine, the tyrosine enters into reaction and this results in the production of a colouration which is a mixture of the shades produced by the two pure amino acids.

According to the assumption of P. Clemens [1899] that the substance capable of forming the most intensely coloured diazo compound has the greatest affinity for the diazo acid, histidine and tyrosine should possess a greater affinity for this reagent than any other amino acids. Furthermore, of the two, histidine must be the one more readily able to combine with the diazo acid, as is proved above.

Hence the golden yellow colouration in the histidine reaction can be obtained even in the presence of many other amino acids without their interfering with the result, provided that only a small quantity of the diazobenzenesulphonic acid just sufficient to combine with the histidine be used. If, however, the diazo acid be added in amount more than equivalent to the quantity of histidine an impure mixed brown or reddish yellow colouration is obtained in the secondary reaction owing to the presence of tyrosine or other amino acids. Hydrogen peroxide may then be added to the solution to obtain the pure lemon yellow, characteristic of histidine, the reddish shade being removed by oxidation.

For the detection of histidine in a protein molecule one of the above mentioned methods can be employed. Although the test works well without the necessity of previously hydrolysing the protein, it is nevertheless better to do so.

Two grams of caseinogen were boiled with 6 cc. of concentrated hydrochloric acid under a reflux condenser for seven hours. The solution, after cooling, was almost neutralised by caustic soda solution and then made alkaline with sodium carbonate solution. It was then diluted with water to 100 cc. The following diazo reactions were carried out with 5 cc. of this solution using N/100 diazobenzenesulphonic acid as reagent.

2 % Hydrolysed caseinogen in cc.	5	5	5	5	5	5
N/100 Diazobenzenesulphonic acid in cc.	1	2	3	4	6	10
Primary colouration	brown	brown	brown	dark brown	dark brown	dark red brown
Secondary colouration	pale yellow	yellow	golden yellow	golden yellow with reddish tint	brown yellow	brown yellow
Colouration after oxidation with 20 % hydrogen peroxide	pale lemon yellow	lemon yellow	lemon yellow	lemon yellow	lemon yellow	lemon yellow

This experiment shows that when the diazo reagent is used in small quantity the secondary colouration is yellow owing to the presence of histidine: as the amount of the reagent increases, the formation of the reddish colour commences and this mixes with the yellow to yield a brown. This phenomenon may be explained by the different degrees of affinity of the amino acids for the diazo reagent, as discussed above. If the pure golden yellow end colouration for histidine be masked by being accompanied by the reddish colouration brought about chiefly by tyrosine, the oxidation process by means of hydrogen peroxide may be used in conjunction with the test, in order to get rid of the interfering tints. In this case a pure lemon yellow colouration of strong intensity indicates the presence of histidine.

I propose to endeavour to apply this modified diazo reaction to the quantitative estimation of histidine in the protein molecule.

Summary.

1. It is easy to distinguish between tyrosine and histidine by means of the diazo reaction by making use of the different colourations produced by reducing their similarly coloured diazo compounds with zinc dust in hydrochloric acid solution, subsequently rendering the solution alkaline with excess of ammonia: tyrosine gives a rose red, histidine a golden yellow colouration.

2. The golden yellow colouration produced by histidine is specific for this amino acid among the protein cleavage products. The reaction may be carried out even with a solution of 1:100,000, but the peculiarly characteristic colour comes into appearance only with a solution stronger than 1:20,000.

3. The rose red colour of tyrosine is not specific for this amino acid, as the same colouration may be produced by some other amino acids, but taking into consideration the fact that the original diazo reaction is given only by tyrosine and histidine, it seems possible to detect the presence of tyrosine by means of these colour reactions. The delicacy of the modified tyrosine reaction is 1:10,000. The reaction has sometimes an advantage over that of Millon as in the case when chlorides are present in large amount.

4. The reddish colourations produced by tyrosine or other amino acids in the secondary diazo reaction are readily destroyed by oxidation with

hydrogen peroxide, resulting in the formation of nearly colourless or faintly rust coloured solutions, while the golden yellow colouration of histidine is little altered, changing only to a lemon yellow.

5. Using a small quantity of diazobenzenesulphonic acid sufficient only to couple with the histidine, or applying the process of oxidation by means of hydrogen peroxide in conjunction with the reduction, the presence of histidine in proteins can be easily detected by reason of the characteristic colouration formed in the secondary diazo reaction.

In conclusion, I wish to express my indebtedness to Professor Hopkins for his valuable advice and criticism and to Mr Foreman for his kindness in supplying me with some of the amino acids used in this investigation.

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XXXIX. THE EFFECT OF ALKALIS ON THE RATES OF OXIDATION AND REDUCTION OF BLOOD.

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(*Received August 24th, 1915.*)

The effect of the addition of acids to blood, in altering the rate at which it can be oxidised or reduced under given conditions, has been studied in this laboratory by Mathison [1911] and by Oinuma [1911], whilst some observations of a similar nature on haemoglobin have been made by Barcroft [1914]. Mathison found that acids accelerated the rate of reduction of blood with a stream of inert gas and that their effect was proportional to the alteration of hydrogen ion concentration produced by them in serum. Oinuma found that, in addition to the acceleration of reduction observed by Mathison, carbonic acid rendered reduced blood less rapidly oxidised by a stream of gas containing oxygen.

No similar observations exist concerning the effect of alkalis. I therefore determined to undertake a research on this subject. The results are given in the following paper.

After testing a number of methods, each of which seemed to promise some improvement on that used by Mathison, I returned to his as being the most satisfactory. Briefly this consists in bubbling gas from a gas holder through a given quantity of blood for a given time. The blood is contained in a tube tilted at an angle of about 15° to the horizontal in a water bath of a given temperature. The tube which I have used has the length of 41 cm. measured from the top to the first bend and the bore is 1 cm. In order to

prevent "frothing," the greatest difficulty presented by this method, a spiral of wire covered with vaseline is inserted into the tube at a convenient distance above the surface of the blood. Sometimes, especially when the concentration of the added alkali is high, the bubbles are not easily broken and the successive films remain unbroken in the tube; thus the streaming of gas is retarded and it is longer in contact with the blood. Accordingly the reduction or oxidation is accelerated at a given rate and duration of bubbling. In order to get uniform results therefore great attention must be paid to this frothing. Slight frothing often makes an experiment useless.

The actual measurement is that of the percentage saturation of the blood with oxygen after gas (either for the purpose of oxidising or reducing the blood) has been passed through for a measured time. The measurements of the percentage saturation have been made with Barcroft's differential blood gas apparatus, the form used being that designed for 1 cc. of blood and described by Barcroft [1914, p. 291]. The shaking of the apparatus has been carried out by the apparatus used by Barcroft for this purpose.

Whilst it is not necessary to go into further detail here as to the apparatus used, I may state the degree of accuracy which it has afforded me. In the present paper there are more than 170 double determinations of the percentage saturation of blood with oxygen. The divergence observed may be tabulated as follows:

Divergence	1 % and under	1–2 %	2–3 %	3–4 %	4–5 %	5–6 %	6–7 %	7–8 %
Number of determinations	43	39	34	18	15	7	3	1

Data given below in the following form:

$$\left.\begin{matrix}42{\cdot}5\\40{\cdot}0\end{matrix}\right\} 41{\cdot}3\ \%$$

signify that gas was bubbled through one sample of blood and that two determinations were made of the resulting blood; but where they are given with a double bracket as follows:

$$\left.\begin{matrix}\left.\begin{matrix}(a)\ 18{\cdot}5\\(b)\ 18{\cdot}2\end{matrix}\right\} 18{\cdot}4\\\left.\begin{matrix}(c)\ 20{\cdot}4\\(d)\ 19{\cdot}3\end{matrix}\right\} 19{\cdot}9\end{matrix}\right\} 19{\cdot}2$$

the meaning is that the blood was divided into two portions. The gas was bubbled through the first, and determinations (*a*) and (*b*) were performed, then in an exactly similar way through the second, (*c*) and (*d*) being the

determinations obtained from it. The divergence between (*a*) and (*b*) therefore is simply that caused by errors in the measurement of the percentage saturation, that between the figures in the second column (18·4 % and 19·9 %) includes the errors due to inequality in the rate of bubbling and similar causes. There are four sets of determinations of this character given below, in none of which do the figures in the second bracket differ by as much as four per cent. from one another.

The blood used was always from animals recently killed without use of any narcotics or drugs. When the blood is not fresh enough the sensitiveness to alkali suffers to a considerable extent. In the case of oxen and sheep the animals were usually killed at the slaughter house about one or two o'clock, in which case the experiment was finished that day. Occasionally the butcher killed the animals late in the evening; the blood was then kept in ice till the next morning. When rabbit's blood was used, the animal was killed just before the experiment; the blood was always defibrinated by whipping.

The addition of alkali to the blood. The same routine has been adopted throughout for the addition of alkali to the blood; 4·5 cc. of blood have been taken and 0·5 cc. of a solution of the alkali in physiological saline (0·9–1 % NaCl) added, the alkali in the portion added being ten times the strength to which it was desired to bring the blood. Thus in the tables, if the blood is described as having alkali added to the extent of M/100 NaOH, 0·5 cc. of M/10 NaOH in physiological saline was added to 4·5 cc. of blood. For each control experiment ("normal blood"), to 4·5 cc. of blood 0·5 cc. of physiological saline was added.

The reduction of the blood was always carried out with commercial nitrogen, this was never quite free from oxygen, containing just under 0·5–0·8 % of that gas. The blood was always thoroughly shaken up with air before the reduction commenced, thus ridding it of carbonic acid and saturating the haemoglobin with oxygen.

The oxidation of the blood was carried out with gas which consisted of approximately 12 % oxygen and 88 % nitrogen; as there were slight differences in the quantity of oxygen present in different experiments, the oxygen sometimes rising as high as 12·6 %, the analysis is given at the head of the tables.

The oxidation experiments have, of course, to be carried out on previously reduced blood. This reduction was effected by the bubbling of nitrogen through the blood for an adequate time. This proved to be 40–50 minutes

at 39–40°, the more alkaline the blood the longer did it take for reduction. A few examples will serve to illustrate the complete reduction.

Blood	Alkali added	Duration of bubbling in minutes	Number of bubbles per min.	Temperature C.	Percentage saturation of blood with O_2 after reduction
Calf	M/200 NaOH	40	60	38°	$\left.\begin{matrix}-1{\cdot}0\\1{\cdot}3\end{matrix}\right\}$ 0·2
Ox	M/50 NaOH	50	72	39°	$\left.\begin{matrix}-2{\cdot}4\\1{\cdot}2\end{matrix}\right\}$ −0·6
Rabbit	M/50 $NaHCO_3$	50	80	39°–40°	$\left.\begin{matrix}0{\cdot}8\\-1{\cdot}2\end{matrix}\right\}$ −0·2

The correction necessary for the exchange of gases in physical solution. In theory this correction differs with each sample of blood. In practice we have applied a uniform correction to our whole series of experiments which is sufficiently accurate for the purpose.

To consider the simplest case. Suppose the blood in the tube at the end of the experiment is completely reduced.

(*a*) 1 cc. of blood will have no oxygen in solution; it will take up from the air in the blood gas analysis apparatus $0{\cdot}031 \times 21/100 = 0{\cdot}0065$ cc. oxygen, since the blood absorbs at 15° about 0·031 cc. of oxygen.

(*b*) It has been in contact with nitrogen at about 710 mm. pressure (that of the atmosphere less the pressure of aqueous vapour at 38°). The amount of gas which it can absorb at 710 mm. pressure and 38° is 0·0103. In the blood gas bottle it is in equilibrium with nitrogen at approximately 600 mm. pressure and at a temperature of about 15°. The amount of nitrogen, which 1 cc. of blood absorbs at 600 mm. pressure and 15° is 0·013. Therefore on shaking up in the blood gas apparatus it gains $0{\cdot}013 - 0{\cdot}0103 = 0{\cdot}00207$ cc. nitrogen. Thus the total amount of gas physically taken up by the blood in the blood gas bottle is $0{\cdot}0065 + 0{\cdot}002 = 0{\cdot}0085$ cc. If the oxygen capacity of 1 cc. blood (diluted with 1/10 of its volume of fluid) be 0·16 cc., then the gas taken up is about 5 % of the oxygen capacity, which must be added as a correction to the observed value of percentage saturation. A similar correction should be made to each experiment for the physical gas exchange.

The Blood Gas of Different Animals.

The blood of different species.

It is clear that the blood of different species of animals presents very different properties both in relation to oxidation and reduction [Barcroft and King, 1909, man and rabbit; Barcroft and Camis, 1909, man and dog].

That some such difference must exist is the necessary corollary to the fact that when in equilibrium with gas of a uniform partial pressure of oxygen the blood of different species exhibits different percentage saturations. Three animals have been studied in this respect, the ox, the sheep and the rabbit. Of these the properties of ox blood and sheep's blood are very similar, but they differ markedly from rabbit's blood which is on the one hand more readily reduced and on the other less readily oxidised. These facts are shown in the following tables:

SERIES 1. *Comparison of ox and sheep's blood, oxidised with* N_2 (87·4 %) + O_2 (12·6 %).

Animal	Duration of bubbling in minutes	Number of bubbles per minute	Temperature C.	Percentage saturation	
Ox	5	60	39	50·2 52·4	51·3
Sheep	5	60	39	53·8 53·2	53·5

SERIES 2. *Comparison of ox and sheep's blood, reduced with* N_2.

Ox	15	60	38	23·5 23·2	23·4
Sheep	15	60	38	26·8 27·6	27·2

SERIES 3. *Comparison of sheep's and rabbit's blood oxidised with* N_2 (88 %) + O_2 (12 %).

Sheep	10	60	38	62·3 60·5	61·4
Rabbit	10	60	38	47·2 45·2	46·2

SERIES 4. *Comparison of sheep's and rabbit's blood, reduced with* N_2.

Sheep	10	60	38	52·2 54·2	53·4
Rabbit	10	60	38	43·0 44·2	43·6

The blood of different animals of the same species.

The fresh blood from different animals of certain species which we have tested presents much the same properties as regards both oxidation and reduction.

Percentage saturation of blood with O_2 after reduction with N_2.

Series	Animal	Duration of bubbling in minutes	Rate of bubbling per minute	Percentage saturation of blood with O_2 after reduction	
1	ox 1	7½	60	49·7 50·9	49·9
		12	60	32·1 33·0	32·6
		10	(interpolated)		39·8
	ox 2	10	60	39·6 39·7	39·7
2	sheep 1	5	72	60·0 60·7	61·7
	sheep 2	5	72	65·2 62·0	63·6
	sheep 1	10	72	40·8 38·8	39·8
	sheep 2	10	72	42·4 38·6	40·5
	sheep 1	15	72	22·8 22·6	24·1
	sheep 2	15	72	28·8 25·4	27·1
	sheep 1	20	72	16·1 —	16·1
	sheep 2	20	72	19·4 13·2	16·3
	sheep 1	30	72	−0·2	
	sheep 2	30	72	0·6 4·6	2·6
3	rabbit 1	10	60	45·1 44·2	44·6
	rabbit 2	10	60	43·0 44·2	43·6

Percentage saturation of blood with O_2 after oxidation with N_2 (= 88 %) + O_2 (= 12 %).

Series	Animal	Duration of bubbling in minutes	Rate of bubbling per minute	Percentage saturation of blood	
1	ox 1	5	72	54·8 51·4	53·1
	ox 2	5	72	52·4 52·8	52·6
	ox 1	10	72	84·5 85·2	84·8
	ox 2	10	72	82·8 80·2	81·5
	ox 1	15	72	94·3 96·0	95·2
	ox 2	15	72	91·7 91·7	91·7
2	sheep 1	5	72	53·8 53·2	53·5
	sheep 2	5	72	50·2 52·4	51·3

However there are many exceptions. Not seldom the blood of animals of the same species has shown a definitely different value of oxygen capacity and also great divergence of percentage saturation with regard both to oxidation and to reduction. It is certain that in man there are slight differences in the percentage saturation of the blood which is in equilibrium with gas containing a uniform partial pressure of oxygen [Barcroft, 1914]. Similar differences have been observed in the blood of different mice with reference to carbon monoxide [Douglas, Haldane, J. S., and Haldane, J. B. S., 1912]. There are no data to show whether these differences are sufficiently large to show themselves in experiments similar to those quoted above.

The Influence of Alkali upon the Reduction and Oxidation of Blood.

Series I. Reduction. The first series of experiments which were rather of a preliminary character consisted in reducing blood made alkaline with NaOH always to the extent of M/200 and varying the circumstances under which the reduction took place, namely, the duration and rate of the bubbling and the animal from which the blood was taken. In every case the more alkaline blood was reduced to a less extent than the control blood to which alkali was not added.

(*a*) Ox blood, 60 bubbles of N_2 per min. at 38°:

Blood	Duration of bubbling in minutes	Percentage saturation after reduction	
Normal	7½	49·7 50·0	49·9
+M/200 NaOH	7½	61·0 59·2	60·1
Normal	12	32·1 33·0	32·6
+M/200 NaOH	12	41·1 43·3	42·2

(*b*) Sheep's blood, 64 bubbles of N_2 per min. at 38°:

Normal	10	41·5 36·3	38·9
+M/200 NaOH	10	45·6 46·7	46·2

(*c*) Ox blood, 60 bubbles of N_2 per min. at 38°:

Blood	Duration of bubbling in minutes	Percentage saturation after reduction
Normal	10	39·6, 39·8 } 39·7
+M/200 NaOH	10	47·5, 49·2 } 48·4
Normal	15	23·5, 23·2 } 23·4; 25·4, 24·3 } 24·9 } 24·2
+M/200 NaOH	15	32·2, 30·0 } 31·1; 33·1, 34·8 } 34·0 } 32·6

(*d*) Sheep's blood, 60 bubbles of N_2 per min. at 38°:

Normal	15	26·8, 27·6 } 27·2
+M/200 NaOH	15	35·0, 30·6 } 32·8

(*e*) Rabbit's blood, 72 bubbles of N_2 per min. at 38°:

Normal	10	21·8, 16·7 } 19·3
+M/200 NaOH	10	29·6, 26·0 } 27·8

(*f*) Sheep's blood, 72 bubbles of N_2 per min. at 38°:

Normal	10	40·1, 37·1 } 38·6; 39·9, 43·6 } 41·8 } 40·3
+M/200 NaOH	10	50·0, 49·1 } 49·6; 50·0, 47·2 } 48·6 } 49·1

Series II. Oxidation. The second series consists of a corresponding set of experiments not on the reduction but on the oxidation of blood. The result shows that blood made alkaline to the extent of M/200 NaOH is more rapidly oxidised than is normal blood.

The gas used for the oxidation in this series consisted of O_2, 12 % + N_2, 88 %:

(*a*) Ox blood, 60 bubbles per min. at 38°:

Blood	Duration of bubbling in minutes	Percentage saturation at end of oxidation
Normal	10	80·1, 79·9 } 80·0
+M/200 NaOH	10	89·1, 88·5 } 88·8

(*b*) Rabbit's blood, 72 bubbles per min. at 38°:

Blood	Duration of bubbling in minutes	Percentage saturation at end of oxidation	
Normal	20	91·8 91·3	91·6
+M/200 NaOH	20	94·3 92·8	93·6

(*c*) Rabbit's blood, 48 bubbles per min. at 39°:

Normal	5	43·7 40·1	41·9
+M/200 NaOH	5	54·8 48·0	51·4

(*d*) Sheep's blood, 48 bubbles per min. at 39°:

Normal	5	41·4 36·7	39·1
+M/200 NaOH	5	48·3 43·3	45·8

The above tables give the percentage saturations attained by the alkaline and normal bloods in a given time. This measurement is of much less theoretical interest than the relative times taken to attain a given percentage saturation, since this latter measurement gives the relative velocities of the two reactions. Some idea of this may be derived in the case of reduction from figures given in Series I. Let V_A/V_N be the ratio of the velocities of reduction of the alkaline and normal blood.

In Series I (*a*), the alkaline blood gives the following data:

	Alkaline blood			Normal blood	V_A/V_N
	Observed		Interpolated	Observed	
Time	7·5	12	9·8	7·5	**0·77**
Percentage saturation	60·1	42·2	50	49·9	
	Normal blood			Alkaline blood	V_A/V_N
Time	7·5	12	9·4	12	**0·78**
Percentage Saturation	49·9	32·6	42·2	42·2	

From Series I (*c*),

	Alkaline blood			Normal blood	V_A/V_N
Time	10	15	12·6	10	**0·78**
Percentage Saturation	48·4	32·6	39·7	39·7	
	Normal blood			Alkaline blood	V_A/V_N
Time	10	15	12·0	15·0	**0·79**
Percentage saturation	39·7	24·2	32·6	32·6	

As the second decimal place in the ratio V_A/V_N is probably valueless, we may conclude that the addition of alkali to the extent of M/200 NaOH reduces the velocity of the reduction to 0·75 – 0·8 of its normal value.

THE COURSE OF REDUCTION AND OXIDATION OF ALKALINE BLOOD.

Reduction. As it seemed desirable to test this result over a much greater range of the whole process of reduction, both in percentage saturation and time, a second series of experiments was performed in which fair curves were obtained representing the course of the reaction from the fifth to the thirtieth minute of the reaction (there is always some uncertainty about the first minute or two as it is not clear that a steady condition has established itself). The results of two experiments are as follows:

EXP. *A*. Sheep's blood. 72 bubbles per minute of nitrogen at 38°:

Time in minutes		5		10		15	
Percentage saturation of blood with O_2 after reduction	Normal	62·7 60·7	61·7	40·8 38·8	39·8	22·8 25·6	24·2
	+M/200 NaOH	70·4 72·2	71·3	48·9 52·6	50·8	32·0 29·5	30·8
Time in minutes		20		25		30	
Percentage saturation of blood with O_2 after reduction	Normal	16·1 —	16·1	10·9 3·0	7·0	−0·2 —	−0·2
	+M/200 NaOH	15·8 19·4	17·6	12·7 10·3	11·5	7·2 3·6	5·4

EXP. *B*. Sheep's blood. Ditto:

Time in minutes		5		10		15	
Percentage saturation of blood	Normal	65·2 62·0	63·6	42·4 38·6	40·5	28·8 25·4	27·1
	+M/200 NaOH	75·0 70·6	72·8	53·7 51·1	52·4	27·8 34·2	31·0
Time in minutes		20		25		30	
Percentage saturation of blood	Normal	19·4 13·2	16·3	—		0·6 4·6	2·6
	+M/200 NaOH	19·1 18·4	18·8	—		5·8 10·2	8·0

Figure 1 is a graphic representation of the data furnished by the first of the two experiments given above, which are very concordant. From it the following values for V_A/V_N may be obtained:

Percentage saturation	60	50	40	30	20
V_A/V_N	0·73	0·73	0·78	0·84	0·84

It is not quite clear from these figures whether the ratio V_A/V_N remains constant in the strict sense of the term over the whole course of the reaction, but clearly it remains approximately so.

Oxidation. To turn to the corresponding data which have been obtained with regard to the relative velocities of the oxidation process in the normal blood and that made alkaline to the extent of M/200 NaOH, the following data enable us to construct curves for the purpose of comparison (Fig. 2).

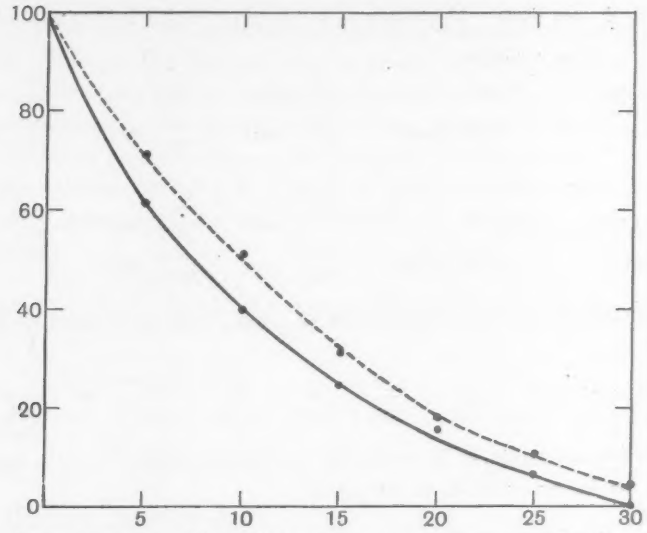

Fig. 1. Rate of reduction of blood with N_2 (Exp. *A*). Ordinates = percentage saturation of blood with O_2. Abscissae = time in minutes. —— normal blood. ..... blood containing M/200 NaOH.

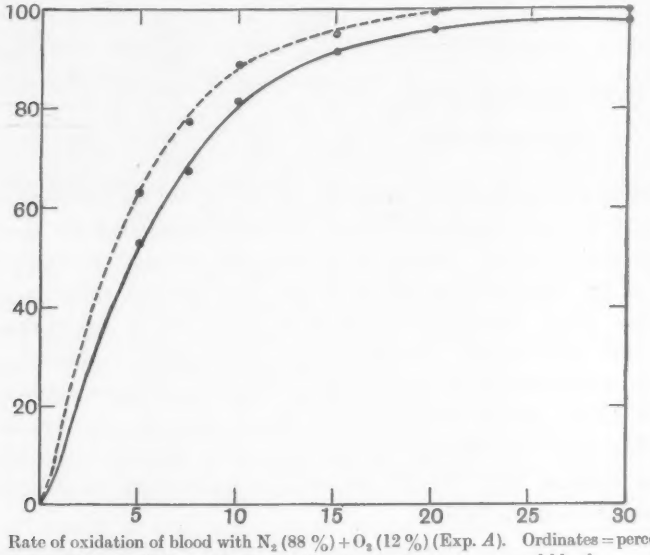

Fig. 2. Rate of oxidation of blood with N_2 (88 %) + O_2 (12 %) (Exp. *A*). Ordinates = percentage saturation of blood with O_2. Abscissae = time in minutes. —— normal blood. ..... blood containing M/200 NaOH.

Exp. *A*. Ox blood. 72 bubbles of N_2 (88 %) + O_2 (12 %) per min. at 38°:

Time in minutes		5		7½		10	
Percentage saturation of blood	Normal	52·4 52·8	52·6	67·8 66·5	67·2	82·8 80·2	81·5
	+M/200 NaOH	61·0 63·7	62·4	79·1 75·0	77·1	90·1 87·7	88·9

Time in minutes		15		20		30	
Percentage saturation of blood	Normal	91·7 —	91·7	96·1 96·1	96·1	97·2 98·8	98·0
	+M/200 NaOH	94·6 95·3	95·0	96·6 102·2	99·4	100·8 —	100·8

The relative velocities of the oxidation process work out from these figures as follows:

Percentage saturation	30	40	50	60	70	80	90
V_A/V_N	1·29	1·29	1·27	1·29	1·29	1·27	1·32

The following data, so far as they go, confirm the value of V_A/V_N obtained above, so far as ox blood is concerned.

Exp. *B*. Ox blood. 72 bubbles of N_2 (88 %) + O_2 (12 %) per min. at 38°:

Time in minutes		5		10		15	
Percentage saturation of blood	Normal	54·8 50·4	53·1	84·5 85·2	84·8	94·3 96·0	95·7
	+M/200 NaOH	68·3 63·0	65·7	92·6 92·0	92·3	101·0 97·0	99·2

from which may be deduced:

Percentage saturation	60	70	80	90
V_A/V_N	1·30	1·27	1·27	1·28

Taking 1·28 as being the mean value of V_A/V_N for the oxidation process and 0·78 for the reduction process it is worth noting that the one is the reciprocal of the other. That this is not necessarily the case is shown by similar data on the effect of temperature on the oxidation and reduction process [Oinuma, 1911]. Bubbling experiments such as are dealt with in this paper show that the lowering of temperature *greatly* retards the reduction process (i.e., the dissociation of oxygen from haemoglobin) whilst it *scarcely* accelerates the oxidation process, indeed if correction is made for the fact that the oxidation curves at different temperatures reach different final equilibrium points [Oinuma, 1911, Fig. 2], the effect of temperature on the oxidation process becomes negligible.

The following consideration, however, prevents us from attaching too much importance to the reciprocal relation existing between the values of

V_A/V_N obtained for the oxidation and reduction processes. In the first place one set of experiments was performed on sheep's blood and the other on ox blood. Later it will appear that the bloods of these two species agree closely in their properties, nevertheless it would have been more satisfactory had the two curves been obtained on the same blood. Moreover there seems to be some evidence that V_A/V_N differs in different species. In the rabbit we get a markedly lower value as regards the oxidation process than in the ox:

Exp. *C*. Rabbit's blood. 60 bubbles of N_2 (88 %) + O_2 (12 %) per min. at 38°:

Time in minutes		5	10	20
Percentage saturation of blood	Normal	41·6, — → 41·6	65·0, 62·5 → 63·8	86·0, 88·1 → 87·1
	+M/200 NaOH	48·2, 47·0 → 47·6	70·0, 72·0 → 71·0	90·0, 89·6 → 89·8

from which may be deduced:

Percentage saturation	60	70	80
V_A/V_N	1·25	1·19	1·17

Similar experiments on calf's blood gave figures of 1·23–1·26 for calf's blood, slightly below that of ox blood, but on this difference we cannot, without further research, lay any stress.

The Effects of Various Concentrations of Alkali on the Reduction and Oxidation of Blood.

Reduction. So far all the experiments of which I have treated have taken the form of contrasting normal blood with that made alkaline to the extent of M/200 NaOH. I have, however, used both stronger and weaker alkalis than this. Thus the effect of M/400 NaOH is quite appreciable.

The effect of alkali does not seem to be the same at all concentrations. Thus in rabbit's blood NaOH seems to have its maximal effect in retarding the reduction of the blood at some concentration of the order of M/50. With concentrations of NaOH higher than this the blood appears to regain its power of reducing, as is shown by the following table in which the percentage saturation of the blood is given after periods of reduction of 10 and 7·5 minutes respectively.

The effect of various concentrations of NaOH
on the reduction of blood.

EXP. *A*. Rabbit's blood. 60 bubbles of N_2 per min. for 10 minutes at 38°:

Blood	Normal		+M/400 NaOH		+M/200 NaOH		+M/100 NaOH	
Percentage saturation	39·7		45·0		49·5		54·8	
after reduction	42·4	41·1	47·3	46·2	52·3	50·9	58·2	56·5
Blood			**+M/50 NaOH**		**+M/33 NaOH**		**+M/20 NaOH**	
Percentage saturation			66·5		55·1		48·3	
after reduction			66·8	66·7	51·3	53·2	43·1	45·7

EXP. *B*. Rabbit's blood. 60 bubbles of N_2 per min. for 7½ minutes at 38°:

Blood	Normal		+M/400 NaOH		+M/200 NaOH	
Percentage saturation	55·4		63·0		67·6	
after reduction	56·0	55·7	61·8	62·4	66·0	66·8
Blood	**+M/100 NaOH**		**+M/50 NaOH**		**+M/33 NaOH**	
Percentage saturation	73·0		80·4		68·9	
after reduction	70·0	71·5	78·7	79·6	71·0	70·0

As the spectroscope shows no alkaline haematin in the blood which contains NaOH to the extent of M/20 (for this confirmation I am indebted to Mr Hartridge) this diminished effect of alkali in higher concentration than M/50 is probably due to the laking of blood which occurs to some extent in the course of reduction with this concentration of alkali.

The fact that increased alkali up to M/100 NaOH retards the rate of reduction of blood with nitrogen is shown also for ox blood by the following figures:

EXP. *C*. Ox blood. 60 bubbles of N_2 per min. for 10 minutes at 38°:

Blood	Normal		+M/200 NaOH		+M/100 NaOH	
Percentage saturation	39·6		47·5		52·7	
after reduction	39·8	39·7	49·2	48·4	53·5	53·1

When sodium bicarbonate is used instead of sodium hydroxide, the data are somewhat different as shown in the following table:

The effect of various concentrations of $NaHCO_3$
on the reduction of blood.

EXP. *D*. Sheep's blood. 60 bubbles of N_2 per min. for 10 minutes at 38·5°:

Blood	Normal		+M/200 $NaHCO_3$		+M/100 $NaHCO_3$		+M/33 $NaHCO_3$		+M/200 NaOH	
Percent. saturation	53·1		54·8		57·0		59·7		60·2	
after reduction	50·2	51·8	52·5	53·7	56·1	56·6	58·5	58·8	59·2	59·9

EXP. *E*. Rabbit's blood. 60 bubbles of N_2 per min. for 10 minutes at 38°:

Blood	Normal		+M/200 $NaHCO_3$		+M/100 $NaHCO_3$		+M/33 $NaHCO_3$		+M/20 $NaHCO_3$		+M/200 NaOH	
Percent. saturation	45·1		49·8		52·3		63·7		68·5		56·9	
after reduction	44·2	44·7	47·1	48·5	50·0	51·2	61·3	62·5	67·7	68·1	52·4	54·7

We see now that the retardation of reduction caused by sodium bicarbonate steadily increases with the increased concentration and, unlike the case of sodium hydroxide, also in higher concentration than M/50, even in M/20.

Oxidation. Increased concentration of alkali seems to increase the power of alkali in accelerating the velocity at which reduced blood is oxidised by gas consisting of nitrogen and oxygen in the mixture which I have used. I have worked up to concentrations equal to M/33. This fact is true not only of NaOH but also of $NaHCO_3$ as is shown by the following table:

The effect of various concentrations of alkalis on oxidation of blood.

Exp. *A*. Sheep's blood. 60 bubbles of N_2 (87·4 %) + O_2 (12·6 %) per min. for 5 minutes at 39°:

Blood	Normal		+M/400 NaOH		+M/200 NaOH		+M/100 NaOH		+M/50 NaOH		+M/33 NaOH	
Percent. saturation	53·8	53·5	59·2	57·9	65·3	63·2	68·0	66·8	73·2	73·6	77·2	75·5
after oxidation	53·2		56·6		61·0		63·6		74·0		73·8	

Exp. *B*. Ox blood. 60 bubbles of N_2 (87·4 %) + O_2 (12·6 %) per min. for 5 minutes at 38°:

Blood	Normal		+M/400 NaOH		+M/200 NaOH		+M/100 NaOH				+M/33 NaOH	
Percent. saturation	49·2	51·3	57·5	56·0	62·0	59·5	60·3	60·8			65·0	68·5
after oxidation	52·4		54·4		57·0		61·3				71·9	

Exp. *C*. Rabbit's blood. 60 bubbles of N_2 (88 %) + O_2 (12 %) per min. for 7½ minutes at 39°:

Blood	Normal		+M/200 $NaHCO_3$		+M/100 $NaHCO_3$		+M/33 $NaHCO_3$		+M/200 Na_2CO_3		+M/200 NaOH	
Percent. saturation	54·7	55·6	59·2	58·4	61·7	60·7	67·4	67·5	62·1	61·7	66·0	63·6
after oxidation	56·5		57·6		59·7		67·5		61·3		61·2	

Exp. *D*. Sheep's blood. 60 bubbles of N_2 (88 %) + O_2 (12 %) per min. for 10 minutes at 39°:

Blood	Normal		+M/200 $NaHCO_3$		+M/100 $NaHCO_3$		+M/50 $NaHCO_3$		+M/200 Na_2CO_3		+M/200 NaOH	
Percent. saturation	63·3	62·4	64·1	64·7	68·2	67·5	74·1	73·5	69·5	68·8	71·5	70·3
after oxidation	61·5		65·2		66·8		72·8		68·2		69·0	

On adding NaOH to the extent of M/33 there is no fall of the power of alkali to accelerate the rate of oxidation of blood as in the case of reduction. I repeated similar experiments several times but could prove no definite fall of the effects of alkali. Any higher concentration of alkali than M/33 caused intense frothing in the tube towards the end of the preliminary reduction of blood, which must last about 50 minutes or more, and made the experiment useless.

The Effect of Different Alkalis on the Rate of Reduction and Oxidation of Blood.

I have compared a number of alkaline salts of sodium, under similar conditions. Of these it appears that sodium hydroxide and sodium carbonate in equimolecular proportion do not differ materially in their effects, and that they are more potent both in retarding reduction and in accelerating oxidation than are either dibasic sodium phosphate or sodium bicarbonate, which in turn have approximately equal effects.

Comparison of the effect of different alkalis of equimolecular concentration upon the rate of reduction.

Exp. *A*. Sheep's blood. 72 bubbles of N_2 per min. for 10 minutes at 38°:

Blood	Normal	+M/200 Na_2HPO_4	+M/200 $NaHCO_3$	+M/200 Na_2CO_3	+M/200 NaOH
Percent. saturation after reduction	39·5 / 41·4 } 40·5	41·2 / 41·1 } 41·2	42·6 / 43·2 } 42·9	45·8 / 45·9 } 45·9	47·8 / 48·5 } 48·2

Exp. *B*. Rabbit's blood. 72 bubbles of N_2 per min. for 10 minutes at 38°:

Blood	Normal	+M/100 Na_2HPO_4	+M/100 $NaHCO_3$	+M/100 Na_2CO_3	+M/100 NaOH
Percent. saturation after reduction	34·3 / 33·8 } 34·1	36·9 / 36·2 } 36·6	41·2 / 39·1 } 40·2	49·4 / 50·5 } 50·0	50·3 / 49·9 } 50·1

Comparison of the effect of different alkalis of equimolecular concentration upon the rate of oxidation.

Exp. *C*. Sheep's blood. 60 bubbles of N_2 (88 %) + O_2 (12 %) per min. for 10 minutes at 39°:

Blood	Normal	+M/200 Na_2HPO_4	+M/200 $NaHCO_3$	+M/200 Na_2CO_3	+M/200 NaOH
Percent. saturation after oxidation	— / 69·1 } 69·1	72·5 / 70·8 } 71·7	72·2 / 71·5 } 71·9	78·0 / 77·1 } 77·6	79·4 / 77·4 } 78·4

Exp. *D*. Rabbit's blood. 60 bubbles of N_2 (88 %) + O_2 (12 %) per min. for 7½ minutes at 38°:

Blood	Normal	+M/100 Na_2HPO_4	+M/100 $NaHCO_3$	+M/100 Na_2CO_3	+M/100 NaOH
Percent. saturation after oxidation	47·4 / 44·0 } 46·2	50·0 / 49·3 } 49·7	50·1 / 51·1 } 50·6	60·0 / 58·2 } 59·1	61·3 / 57·1 } 59·2

The Interference of Carbon Dioxide with the Effect of Alkali on the Rate of Oxidation of Blood.

As shown by Bohr, Hasselbalch and Krogh [1907] and also by Mathison [1911] and Oinuma [1911], the presence of carbon dioxide hastens the rate of reduction of blood and slows that of oxidation. We see now that alkali acts in just the opposite way. The last series of the present investigation was undertaken to see the extent to which carbon dioxide and alkali counteract each other with regard to the oxidation of blood. As oxidising gas two mixtures were employed at body temperature: each consisted of nitrogen containing 12·8 % oxygen, but one with 5·6 % CO_2 added, the other without CO_2. The partial pressures of oxygen and carbon dioxide in the former roughly correspond, at body temperature and with the tension of aqueous vapour corrected, to 100 mm. and 40 mm. of mercury respectively; the mixture is therefore approximately equivalent to alveolar air. The following data were obtained after passing a stream of each gas mixture through normal and alkaline blood which had been previously entirely reduced.

Interference of CO_2 with the effect of alkali upon the rate of oxidation.

Exp. *A*. Rabbit's blood. 60 bubbles of gas mixture at 39° for 10 minutes:

	Percentage saturation of blood		
Gas mixture	Normal	+M/100 $NaHCO_3$	+M/100 NaOH
N_2 (87·2 %) + O_2 (12·8 %)	63·5, 64·0 — 63·8	75·5, 68·0 — 69·3	74·7, 72·3 — 73·5
N_2 (81·6 %) + O_2 (12·8 %) + CO_2 (5·6 %)	56·2, 55·0 — 55·6	61·9, 58·8 — 60·9	67·3, 64·0 — 65·7

Exp. *B*. Rabbit's blood. 60 bubbles of gas mixture at 39° for 10 minutes:

	Percentage saturation of blood		
Gas mixture	Normal	+M/200 NaOH	+M/100 NaOH
N_2 (87·2 %) + O_2 (12·8 %)	64·7, 67·0 — 65·9	73·7, 71·8 — 72·8	75·1, 77·3 — 76·2
N_2 (81·6 %) + O_2 (12·8 %) + CO_2 (5·6 %)	57·1, 55·6 — 56·8	65·8, 63·7 — 64·8	68·5, 66·3 — 67·4

From these results it is obvious that blood made alkaline by adding M/100 NaOH is oxygenated to a greater degree by a gas containing 5·6 % CO_2 than normal blood is oxidised by a similar gas which contains no CO_2, and blood with NaOH added to M/200 to a slightly smaller degree. Thus the depressing action of CO_2 at the same partial pressure as in the alveolar air

on the rate of oxidation of blood is counteracted by the addition of NaOH to the blood to some concentration between M/200 and M/100. The addition of $NaHCO_3$ to M/100 does not wholly counteract the effect of CO_2 of the percentage mentioned above.

If we compare the figures in any one line, we find another fact worthy of mention, i.e., the differences between the percentage saturation of normal blood and that of alkaline blood are practically equal in the cases in which the oxidising gas contained CO_2 and in those in which no CO_2 was contained. Accordingly it may be asserted that the alkali exerts an accelerating effect of about equal extent on the oxidation of blood both in cases where the oxidising gas used contains CO_2 and where no CO_2 is present.

It is clear that these results concerning the effect of alkali on the gas exchange of blood have a certain clinical significance. Numerous observers, Poulton and Ryffel [1913], Peabody [1914], Lewis, Barcroft and their collaborators [1913], have described acid conditions of the blood associated with dyspnoea probably of renal origin. Such conditions might conceivably be treated by the addition of alkali in some form or other to the blood; the line of argument being (1) that the alkali by reducing the hydrogen ion concentration of the blood would relieve the dyspnoea; (2) that it might be added in sufficient quantity to do this in spite of a possible rise in the CO_2 occasioned by the slower respiration. The results given show that the alkali would assist the taking up of oxygen in the lung and would probably not retard the reduction process, in the case of patients resting in bed, sufficiently to impair the respiration in the tissues to an extent which would be really injurious.

Summary.

(1) The rate at which arterial blood gives up its oxygen at low oxygen tensions or the venous blood takes up the gas at high oxygen tensions is influenced to an appreciable degree by the presence of alkali in small quantity. The oxygenation is hastened and the reduction slowed.

(2) The addition of alkali to the extent of M/200 NaOH reduces at body temperature the velocity of reduction of blood on an average to 0·78 of its normal values and increases that of oxidation approximately to 1·2–1·3. Of these two values one is nearly the reciprocal of the other.

(3) The above effect of alkalis (NaOH, $NaHCO_3$) upon the rate of oxidation and reduction increases with increased concentration up to about M/50.

The power of NaOH in retarding the velocity of reduction appears to fall at a concentration higher than M/50.

(4) In equimolecular concentrations, NaOH and Na_2CO_3 have an equivalent effect. The same is true of Na_2HPO_4 and $NaHCO_3$ but their effect is less than that of NaOH and Na_2CO_3.

(5) In the rabbit the effect of carbon dioxide, which is contained in the oxidising gas in such a proportion as is found in the alveolar air, upon the rate of oxidation of blood is counteracted by the addition of NaOH of concentration between M/200 and M/100.

(6) Blood of animals of different species is reduced or oxidised at different rates. In the same species the rate of reaction is generally similar in different animals. There are, however, many exceptions.

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XL. THE PART PLAYED BY THE DIFFERENT BLOOD ELEMENTS IN GLYCOLYSIS.

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When blood is allowed to stand at a suitable temperature an appreciable amount of the sugar present disappears. This glycolysis occurs to a marked extent in human blood; it is also observed in the blood of certain of the lower animals (dog, sheep) [Loeb, 1913], while in other animals (pig) [Melvin, 1912], (goat) [Maclean, 1911], little or no decrease in sugar content appears to take place[1]. This difference explains certain divergent statements encountered in the literature. Not only are there differences in the degree of glycolysis in different animals, but great variations may be observed in the same animal under different circumstances. In certain pathological conditions in man, as will be shown later, glycolysis may be greatly increased.

Various observers have demonstrated that the glycolytic power of blood is essentially dependent on the formed tissue elements, and that the fluid constituent plays no part [Rona and Döblin, 1911; MacLeod, 1913]. In general, the power of destroying sugar has been ascribed to the red corpuscles and various theoretical deductions have been drawn, based chiefly on the normal glucose content of the erythrocytes and the relative inability of glucose to penetrate the envelopes and gain access to their interior [Loeb, 1913]. Rona and Arnheim [1913] attempted to separate the cellular elements of blood into erythrocytes and white cells, and found, on estimating the glycolytic

[1] Possibly, this lack of glycolytic power in certain animals is more apparent than real. The presence of a small amount of some sugar-forming substance such as glycogen might supply sugar to replace that destroyed, so that estimation of the sugar content before and after incubation would give no real clue to the glycolytic activity of the blood.

activity of the two constituents, that no glycolysis was produced by the white cells while the red cells were distinctly active. Levene and Meyer [1912], however, showed that leucocytes have the power of destroying glucose, at least in the presence of phosphate mixture. Rona and Arnheim then added phosphates to their white cells, and found that this addition played an important part, the white cells now showing marked glycolytic activity.

So far, the evidence pointed to the red cells as the chief agent in the phenomenon in normal blood, but, since the discovery of Levene and Meyer mentioned above, certain observers have assumed, without any real evidence, that glycolysis depends chiefly or entirely on the leucocytes.

The chief obstacles in the way of settling this question were bound up with the difficulty of separating the blood elements into white and red cells; a suitable method for accurately estimating sugar in small amounts of blood was also needed.

In recent years several methods for this purpose have been published but certain of them are unsuitable. One of the best is Bang's micro-method. In the following experiments, a modification of the method described by Gardner and MacLean [1914] has been adopted. The objects aimed at were to avoid filtration of the cuprous oxide, and to increase the readings given by very small amounts of sugar so that very minute differences could be detected and estimated. The method will be fully described in another paper. It is sufficient to mention here, that such a small amount of sugar as 0·1 of a milligram gave a reading of nearly 1 cc. of a standard thiosulphate solution. From this it is obvious that very small differences in sugar content could be easily detected, and that very small amounts of blood sufficed for the determinations. In each experiment 1 cc. blood was used. Contrary to the results of Rona and Arnheim [1913], we find that the leucocytes play a very important role in glycolysis, being very much more active than the red corpuscles; on the other hand our experiments agree with the results of these and other authors who maintain that the red corpuscles are also capable of causing glycolysis. Though the red corpuscles are active in this respect, each red corpuscle is of little importance when compared with a white corpuscle; the very much greater number of red corpuscles in normal blood, however, tends to correct this predominance of the white so that in reality the red cells do account for a good deal of the total glycolysis of blood. In abnormal bloods, such as that of patients suffering from leucocythaemia, the part played by the white cells is very marked as is shown in the following experiment.

Experiments with Pathological Blood.

Experiment I.

Patient suffering from leukaemia was bled, and to 25 cc. blood 0·1 % potassium oxalate added. The blood was centrifuged and the plasma removed. The upper layer, which contained an excess of leucocytes, was now carefully removed, mixed with plasma, again centrifuged and the upper layer used for glycolysis (*Leucocyte Fraction*). By means of a very fine pipette the lower layers, containing an excess of red cells, were removed mixed with plasma and again centrifuged. The lower layers of this were then taken and used for glycolysis (*Red cell Fraction*).

To each fraction some glucose was added: 1 cc. of each was then taken and the sugar content estimated; both were incubated at 37° for 1·75 hours and the sugar again estimated.

Blood counts of the number of red and white cells in each fraction were made. From the results obtained it is obvious that the fraction containing excess of leucocytes was very much more active than the red cell fraction, which contained but a comparatively small number of leucocytes.

	Before incubation		After incubation			Blood counts	
Blood fraction used	No. of cc. of N/250 thiosulphate used	Sugar present per 100 cc. in g.	No. of cc. of N/250 thiosulphate used	Sugar present per 100 cc. in g.	Sugar destroyed per 100 cc. in g.	Red cells per c.mm.	White cells per c.mm.
Leucocyte fraction	11·75	0·132	4·85	0·054	0·78	6,000,000	231,200
Red cell fraction	12·05	0·134	9·55	0·106	0·28	10,000,000	11,360

Here the activity of the leucocyte fraction was very marked, sugar equivalent to 6·90 cc. N/250 thiosulphate being used up in 1·75 hours, while the red cell fraction, which contained four millions more of cells than the white cell fraction, destroyed sugar only equivalent to 2·50 cc. N/250 thiosulphate.

Part of the original blood was also tested; it contained 3,500,000 red cells and 81,000 white cells per c.mm.

In 1·75 hour it used up sugar equivalent to 3·3 cc. N/250 thiosulphate solution—a greater amount would be found in the case of a normal blood containing five to six million red cells. Similar results to the above were found in other cases. The figures in one more case are given and they will suffice to show that in leukaemic blood the increased leucocyte content plays a marked part in the phenomenon of glycolysis.

Experiment II.

The blood was divided into the two fractions—white and red cells—as in Experiment I. Incubation here lasted for two hours.

	Before incubation		After incubation			Blood counts	
Blood fraction used	No. of cc. of N/250 thiosulphate used	Sugar present per 100 cc. in g.	No. of cc. of N/250 thiosulphate used	Sugar present per 100 cc. in g.	Sugar destroyed per 100 cc. in g.	Red cells per c.mm.	White cells per c.mm.
Leucocyte fraction	5	0·055	none	none	0·055 +	5,000,000	212,400
Red cell fraction	4·5	0·050	1·55	0·017	0·033	10,000,000	17,640

In this experiment the amount of sugar added was too small and the incubation period rather long, for all the sugar was used up in the leucocyte fraction. The numbers obtained for this fraction are therefore too low, for if more sugar had been present more would have been destroyed. While showing the marked effect of the leucocytes these experiments also prove that the red cells take an active part in glycolysis.

They also show that the addition of phosphates is not necessary to bring about this effect. Since the above experiments were done with pathological blood, it might be argued that no deductions applicable to normal blood could be drawn from the results. Nearly twenty experiments, however, have been carried out on normal blood and in every case it has been found that while both red cells and leucocytes are active, the leucocytes are cell for cell very much more active than the red cells. The exact glycolytic power of the white cell compared with the erythrocyte varies very much indeed, but in a general way the experiments suggest that each white cell is from 200 to 1000 times as active in glycolysis as a red cell. Seeing that many delicate manipulations are necessary in arriving at these results it is probable that no great importance is to be attached to the exact figure found for the red to white ratio in any given experiment; it is merely to be regarded as something of the order of the number given. When, however, it is found that in every experiment the glycolytic value for white cells comes out enormously greater than that for the red cells the part played by the leucocytes is abundantly demonstrated. No doubt the destruction of sugar caused by the red cells may depend on certain conditions already mentioned such as the amount of sugar inside the cell, and on other factors: probably also the activity of the white cell varies very much in different cases. If we assume that the white cell is on an average 1000 times as powerful a glycolyser as a red cell, and that normal blood contains about five hundred times as many red cells as white, the leucocytes in a sample of such a blood would account for the destruction of twice as much sugar as the red cells.

Experiments with Normal Blood.

We found it quite impossible to separate the blood into two fractions—one containing white cells only, and the other red cells. Invariably our red cell fraction contained leucocytes and our leucocyte fraction had always large amounts of red corpuscles. It is obvious, however, that complete separation into two constituents is not necessary. All that is required in order to determine the relative action of the red and white blood cells on glycolysis is to divide the blood into two fractions in which, though both constituents are present, they differ greatly in number. The sugar in each part is estimated before and after incubation. This at once gives conditions from which the effect of either constituent can be worked out by a simple algebraical equation. The following experiments illustrate these points.

Experiment III.

12 cc. of blood were obtained from a student by inserting a needle in a vein, coagulation being prevented by the addition of 0·1 % potassium oxalate. The blood was centrifuged, the plasma separated and the top layer pipetted off. This layer constituted the leucocyte fraction, while the lower layer represented the red cell fraction. The sugar was estimated in each fraction before and after incubation for 4 hours at 37°.

	Leucocyte fraction	Red cell fraction
Sugar before incubation in g. per 100 cc.	0·037	0·040
Sugar after incubation in g. per 100 cc.	0·009	0·006
Total sugar used in g. per 100 cc.	0·028	0·034
Blood count in cells per c.mm.	Red cells 6,000,000 White cells 6,960	Red cells 8,000,000 White cells 4,440

Ratio of activity of leucocyte to red cell = 178 : 1.

Also it is evident that both red and white cells must have been active, for, though the white cell is much more active than the red, if the glycolysis depended only on the white corpuscles, the leucocyte fraction should have destroyed much more total sugar than the red cell fraction.

Experiment IV.

Blood from a patient was treated exactly as in Experiment III with the exception that incubation was restricted to 2 hours at 37°.

	Leucocyte fraction	Red cell fraction
Sugar before incubation in g. per 100 cc.	0·033	0·032
Sugar after incubation in g. per 100 cc.	0·010	0·013
Total sugar used in g. per 100 cc.	0·023	0·019
Blood count in cells per c.mm.	Red cells 7,200,000 White cells 15,520	Red cells 9,800,000 White cells 2,240

Ratio of activity of leucocyte to red cell = 364 : 1.

Five other experiments were carried out in exactly the same way except that in certain of them a small amount of glucose was added. The results are given in the table. In No. 8 the red cell fraction first obtained was again centrifuged and the bottom layer of the latter used as the red cell fraction.

No.	Source of Blood	Amount of blood taken in cc.	Sugar added in mgrs.	Time of incubation in hrs.	Sugar of leucocyte fraction: Percent. before incubation	Sugar of leucocyte fraction: Percent. after incubation	Total sugar destroyed by leucocyte fraction per 100 cc.	Sugar of red cell fraction: Percent. before incubation	Sugar of red cell fraction: Percent. after incubation	Total sugar destroyed by red cell fraction per 100 cc.
V	student	15	none	1·5	0·03	0·009	**0·021**	0·028	0·01	**0·018**
VI	patient	15	15	2	0·091	0·061	**0·030**	0·083	0·058	**0·025**
VII	student	20	15	3	0·097	0·056	**0·041**	0·088	0·052	**0·036**
VIII	patient	15	10	1·75	0·084	0·062	**0·022**	0·072	0·058	**0·014**
IX	student	15	10	—	0·062	0·031	**0·031**	0·063	0·049	**0·014**

The "blood counts" of the above experiments gave the figures per cubic millimetre.

	Leucocyte fraction	Red cell fraction
No. V	Red cells 9,200,000 White cells 10,440	Red cells 12,500,000 White cells 3,600
No. VI	Red cells 4,700,000 White cells 13,040	Red cells 10,700,000 White cells 3,840
No. VII	Red cells 4,500,000 White cells 12,480	Red cells 10,000,000 White cells 3,840
No. VIII	Red cells 8,200,000 White cells 23,000	Red cells 10,000,000 White cells 1,280
No. IX	Red cells 5,000,000 White cells 29,320	Red cells 11,800,000 White cells 1,000

	Ratio of leucocyte activity to red cell activity
No. V	863 : 1
VI	965 : 1
VII	850 : 1
VIII	358 : 1
IX	779 : 1

It is obvious from these experiments that the leucocyte is in every case much more active than the red cell in the production of glycolysis.

Glycolysis in Haemolysed Blood.

If, according to the observations of Loeb and others, the power of destroying sugar resided entirely in the red cell it is difficult to understand the results obtained by Edelmann [1912], who showed that laking of the red cells by saponin does not destroy the glycolytic power. It is known, however, that it is possible under certain conditions to lake the red cells without destroying the leucocytes, and it may possibly be that in these experiments where the red corpuscles were destroyed, the white corpuscles were left sufficiently active partially to destroy the sugar present.

In all the experiments already given, the blood was centrifuged in its own plasma. In some later experiments, some small differences in manipulation were introduced and the corpuscles were washed with isotonic saline solution. Here the results were in agreement with those obtained with unwashed corpuscles except that the ratio of leucocyte activity to that of the red cell was somewhat changed. In all cases clotting was prevented by the addition of 0·1 % potassium oxalate solution.

Experiment X.

The blood was obtained from a student in the usual manner. To this 20 mgrs. sugar were added; after centrifuging, the plasma was removed and the top layer of the solid separated; this was shaken up with the separated plasma and again centrifuged. The top layer of this was mixed with normal saline, again centrifuged and the top layer again pipetted off and used for the experiment (= leucocyte fraction). After the first centrifuging of the blood the layer left behind after removal of the leucocytes was mixed with some normal saline. This was then centrifuged, the lower part of the solid layer formed removed by a fine pipette, again mixed with normal saline, and centrifuged. The lower layer of this, which contained only a relatively small number of white cells, represented the red cell fraction. Incubated 1·5 hours.

	Leucocyte fraction	Red cell fraction
Sugar before incubation in g. per 100 cc.	0·0810	0·0792
Sugar after incubation in g. per 100 cc.	0·0468	0·0567
Total sugar destroyed in g. per 100 cc.	0·0342	0·0225
Blood count in cells per c.mm.	Red cells 7,725,000 White cells 23,520	Red cells 8,275,000 White cells 840

Ratio of activity of white to red cell = 218 : 1.

Experiment XI.

This was carried out exactly as in Experiment X. The blood was obtained from a healthy soldier. To 20 cc. blood, 20 mgrs. sugar were added.

	Leucocyte fraction	Red cell fraction
Sugar before incubation in g. per 100 cc.	0·091	0·078
Sugar after incubation in g. per 100 cc.	0·052	0·062
Sugar destroyed in g. per 100 cc.	0·039	0·016
Blood count in cells per c.mm.	Red cells 8,225,000 White cells 42,160	Red cells 8,625,000 White cells 1,120

Ratio of activity of white to red cell = 325 : 1.

In these experiments it will be noticed that the activity of the white corpuscle compared with the red cell is low. This however is apparently not due to the action of the saline, as the next experiment shows.

Experiment XII.

20 cc. blood from student treated exactly as above. Incubation 1·75 hours. 20 mgrs. sugar added.

	Leucocyte fraction	Red cell fraction
Sugar before incubation in g per 100 cc.	0·138	0·091
Sugar after incubation in g. per 100 cc.	0·110	0·078
Sugar destroyed in g. per 100 cc.	0·028	0·013
Blood count in cells per c.mm.	Red cells 6,000,000 White cells 17,600	Red cells 10,000,000 White cells 1,800

Ratio of activity of white to red cell = 1149 : 1.

Several experiments were carried out and always with the same general result, though the figures obtained varied considerably. The results given serve to establish the activity of both red and white corpuscles in the process of glycolysis.

Conclusions.

(1) Both the red and white cells of normal blood take part in glycolysis.

(2) The white cells are much more active than the red; the ratio of activity of the white to the red cell varies roughly from about 200:1 up to 1000:1.

(3) In leukaemic or other blood containing excess of leucocytes, glycolysis is much more active than in normal blood.

(4) In normal blood it is probable that the comparatively small number of leucocytes present exert a somewhat greater influence on the total glycolysis than do the red cells which are present in relatively large amount.

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